HUMAN AND MURINE SALIVARY GLAND GROWTH *IN VITRO* WITH PARTICULAR EMPHASIS ON DUCTAL EPITHELIAL CELL GROWTH

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by

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PREFACE

The work in this thesis was undertaken in the Department of Oral Medicine and Pathology in the University of Glasgow Dental School during the period July 1977 to August 1981 when the author was employed as a Research Assistant. The work was supported by a grant from the W.J. Moore Trust.

Some of the techniques used in this thesis are modifications of previously published work and some are original techniques developed by the author in conjunction with the other members of staff in the Department of Oral Medicine and Pathology.

The applications of these techniques as described in the present study were undertaken by the author personally. Tissue microtomy and preparation for electron microscopy was carried out by the technical staff under the direct supervision of the author.

The studies described in this thesis are either entirely original or greatly extend previously reported investigations.

Parts of the work of this study have been presented at scientific meetings and have been published some, as abstracts, in the Journal of Dental Research*. These include the following :-

A. Papers Presented at Scientific Meetings

1.* "Quantitative in vitro growth studies on newborn (CFLP) mouse submandibular, sublingual and parotid salivary glands". International Association of Dental Research (British Division) London, April 1978 (with M.M. Ferguson).

2.* "The Identification of cells from the in vitro tissue culture of newborn mouse salivary gland and human labial gland".
International Association of Dental Research (British Division) London, April 1978 (with W. Marshall and M.M. Ferguson).

3. "Growth Characteristics and cell populations in in vitro salivary gland culture".
European Tissue Culture Society, Glasgow, July 1979 (with W. Marshall and M.M. Ferguson).

4.* "Effect of neurotransmitters on murine salivary gland cultures in vitro".
International Association of Dental Research (British Division) Sheffield, April 1979 (with M.M. Ferguson and W. Marshall).

5. "Quantitative response of murine salivary glands in vitro to selected neurotransmitters".
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B. Publications

1. "A modified autoradiography technique applicable to coverglass cell cultures".
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2. "Murine submandibular salivary gland growth characteristics in vitro utilising cyanocrylate-aided adhesion to glass".
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3. "Quantitative studies on growth and cell population identification in murine salivary gland cell culture".
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5. "Study of the growth and ductal epithelial cell component of human labial salivary glands in vitro." Archives of Oral Biology, 1982 (In Press) (with M.M. Ferguson, W. Marshall, M. Wallace and D. Wiesenfeld).

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ABSTRACT

The mechanism by which drug-induced salivary gland enlargement occurs clinically is not known. Denervation experiments suggest a direct cellular action of the relevant compounds in the presence of intact cell receptors. The denervated gland enlarges *in vivo* in response to exogenous isoprenaline. Since the salivary glands produce cell growth factors a complex series of events may be involved. Target cells for neurotransmitter action and for growth factor production have not been isolated.

This thesis describes an alternative approach to understanding drug-induced salivary gland enlargement. By using cultured cells the aim was to isolate the salivary cells from the complexities of *in vivo* control.

However, it was first necessary to define the culture requirements of the three major murine and human labial salivary glands. Conditions were designed to optimise growth from primary explant cultures and to facilitate identification of cells in the outgrowth. An optimum medium composition was established for growth of salivary gland cells on different substrata. The cells remain viable by Trypan Blue exclusion and can be serially propagated.

Cell identification was by morphology, ultrastructure, histochemical criteria and by cell-directed antibodies. Electron micrography confirmed that some of the cultured cells were epithelial in character. By light microscopy cell morphology bore an inconstant relationship to other markers for ductal epithelial cells. Similar proportions of cells react positively for 11β hydroxysteroid dehydrogenase and with salivary duct antibody. Either criterion appears to quantify the proportion of ductal epithelial cells in a mixed culture.

Quantification of the cells growing from human salivary explants was also attempted. Ductal cells convert cortisol to cortisone but there was no direct relationship observed between 11β hydroxysteroid dehydrogenase and the metabolism of cortisol.

The main conclusions are that human and murine salivary gland cells can be propagated *in vitro* for prolonged periods and that a proportion of these cells are ductal in origin. Isoprenaline in a wide range of concentrations *in vitro* did not sufficiently alter the salivary cell growth patterns to account for gland enlargement *in vivo*. Other neurotransmitters even at high concentrations produced no increase in culture growth. Under these circumstances all neurotransmitters, except isoprenaline, altered the proportion of ductal cells present.

GENERAL INTRODUCTION

From animal experiments, particularly in rodents, a great deal is known about the factors regulating the gross glandular development of salivary glands, but very little is known about factors controlling salivary gland growth and differentiation. Experiments into the factors involved in growth and differentiation would be difficult to perform *in vivo* and the complexity of the possible interaction of a number of factors, as well as ethical considerations, limit the kind of information that can be obtained from such experiments. However, using an *in vitro* system many variables can be easily controlled and growth effects attributable to single agents can be deduced.

Of primary importance in this work was the investigation of which variable factors in the cell environment *in vitro* determined the amount of growth observed. Any growth which did occur then had to be quantified and the system employed had to be reproducible, non-invasive and allow the continued viability of the salivary gland explant and any cellular outgrowth which occurred from it. By being non-invasive and allowing continued cell viability, sequential measurements of growth could be made.

Salivary glands are epithelial in origin and have a mesenchymal component associated with them in the form of septae and a gland capsule if present. *In vitro*, at least two main cell populations would still be present and identification of each is important if the effects of variations in cell environment as well as of neuro-transmitters are to be analysed in terms of individual cell populations. The validity of using cell morphology, ultra-structural morphology, cell function and surface antigen characteristics in the identification of epithelial cell populations has had to be determined.

The aims and design of the present study can be summarised as follows :-

In Chapter 1 the general concepts of salivary gland growth and differentiation *in vitro* are presented. The literature concerning salivary gland organ culture is large but the number of publications relevant to salivary gland cell culture is limited. Chapter 1 therefore includes a general review of this literature and an evaluation of the current concepts governing salivary gland growth and differentiation *in vitro* and the relationship to salivary gland function *in vitro*.

The development, structure and ultrastructural features of murine major and human minor salivary glands are outlined in Chapter 2. The ultrastructural aspects of salivary gland innervation as it may relate to the expected response to neurotransmitters has been reviewed.

In Chapters 2, 3 and 4 the role of salivary gland growth factors and neurotransmitters are reviewed. The former group of growth factors may illustrate an endocrine as well as an exocrine function for the submandibular salivary glands. The relationship between neurotransmitters as they affect salivary gland growth and differentiation *in vivo* and *in vitro* is also discussed.

In Chapter 5 the source of solutions, materials and methods used in the study are presented. Techniques developed during this study such as some autoradiographic procedures are given in detail.

In order to evaluate the effect of any agent on salivary gland growth and cell populations, a normal range of growth under defined conditions must be established. In Chapter 6 the variable parameters which may influence salivary gland growth have been defined and their effects quantified. Having defined the optimal growth conditions the response to selected neurotransmitters could then be investigated.

The primary aim of the study was to develop a suitable *in vitro* system for the quantification of the epithelial components of human and murine salivary gland growth. Very little is known of normal salivary gland epithelial cell growth *in vitro* since techniques for identifying cells of epithelial origin have been limited. In Chapter 7 the techniques developed for identifying murine ductal epithelial cells are presented.

Chapter 8 describes experimental studies on murine salivary glands in which the effect of selected neurotransmitters on epithelial cell populations has been evaluated. The data so derived allows some speculation as to the intrinsic control mechanisms of ductal epithelial cell differentiation.

In Chapter 9 the culture techniques developed to study murine salivary gland growth have been applied to human labial salivary glands and expanded to encompass other immunological adjuncts to identifying epithelial cell populations. In addition, non-invasive attempts to quantify the ductal epithelial cells present are presented. The ultimate aim of this part of the study is to develop a system whereby factors of possible aetiological and prognostic significance in salivary gland disease processes could be investigated.

Chapter 10 presents a general discussion on experimental findings with consideration of the problems involved in the study of cell proliferation in the epithelial component of salivary tissues.

CHAPTER 1

SALIVARY GLAND GROWTH IN VITRO

1.1 INTRODUCTION

Animal tissue culture is a term which specifically refers to the maintainance *in vitro* of discrete tissue fragments for more than 24 hours whilst not necessarily maintaining their structural integrity (Federoff, 1967 ; Paul, 1975). It has come to encompass both cell culture and organ culture. Cell culture and organ culture refer respectively to the *in vitro* maintainance of cells and tissues with the latter emphasising the preservation of the structural and functional integrity of the tissue. This study investigates principally cell culture aspects of the growth of salivary glands.

Tissue culture began in the mid-1900's (Vulpian, 1859 ; Roux, 1885), but the conclusions from these experiments that tissues would grow *in vitro* did not begin to be realised until the work of Harrison (1907, 1908) and Carrel (1912). Carrel's work on chick muscle was extremely important because although Harrison, working on frog embryo fragments, demonstrated that organised growth occurred *in vitro* for a limited time, it was Carrel who by the simple expedient of feeding the tissues demonstrated that prolonged viability *in vitro* was possible. This finding conflicted with the previously held view that cells were intrinsically unable to grow for long in culture.

Investigation of human tissues began with Ljunggren (1898) using human skin followed by Thomson and Thomson's (1914) work on human tumours. The latter authors considered their attempt at organ culture disappointing since cell outgrowth still occurred. The classical studies undertaken later on the developing limb-bud of the chick succeeded in intentionally maintaining tissue structure *in vitro* as close as possible to the *in vivo* situation (Strangeways and Fell, 1926 ; Fell and Robison, 1929). This work, which Grobstein (1953a) later adapted and applied to mouse

salivary glands, produced a great deal of information concerning morphogenesis and epithelial-mesenchymal interactions.

Carrel's work had shown that adequate nutrients were required for continued cell viability which it then became possible to study. Consequently, in parallel with these developments work on culture media was progressing. The disadvantages of the early clot type culture with wholly natural extracts were that the composition of the medium was unknown and thus potentially subject to great variation, and that the explant itself digested the clot. One of the earliest attempts to investigate the factors in the medium necessary for growth and survival was that of Lewis and Lewis (1911 a,b). This type of investigation has undergone continuous modification by a large number of workers (Willmer, 1965) resulting in the current complex synthetic media frequently eponymously linked to their originators. Many mammalian cells will only grow if the synthetic medium also contains varying proportions of supplements such as serum. For foetal calf serum this growth enhancing property has been claimed to be a single protein, fetuin (Fisher, Puck and Sato, 1958).

Much of the initial work on tissue explants was with tissues easily accessible and thus potentially subject to bacterial contamination. For example skin culture techniques (Medawar, 1948, Reaven and Cox, 1968) would have been impossible were it not for the introduction of antibiotics. It was fortunate that earlier work had been carried out for example by Carrel who was a surgeon familiar with aseptic techniques. Even with the introduction of antibiotics, and now antifungal agents, the basis of tissue culture work depends on meticulous attention to aseptic techniques.

Salivary gland culture has only been studied in detail in the last thirty years yet has provided a wealth of knowledge about these organs. The application of <u>in vitro</u> techniques to salivary glands is best illustrated by considering molecular aspects of salivary gland development.

1.2 DIFFERENTIATION OF SALIVARY GLAND CELLS

The cellular composition of the salivary glands consists of acini and duct cells derived from oral epithelium surrounded by a variable myoepithelial element. Fibrous tissue septae separate the glands into lobules and major salivary glands have a mesenchymally derived capsule.

The application of *in vitro* techniques to the study of salivary gland growth and differentiation has resulted in great advances in our knowledge of both these processes. *In vitro* techniques have permitted separation, usually by enzymatic digestion, of the epithelial component of the salivary gland from the mesenchymal component ; by performing recombination experiments, epithelial-mesenchymal interactions can be studied and their relationship to glandular assembly established.

Identification of the epithelial constituent and the connective tissue thickening was by inspection at the appropriate rudimental stage.

Borghese (1950a) working predominantly on the foetal submandibular salivary gland of the common mouse, Mus musculus, showed that the rudiment of the submandibular salivary gland would develop almost normally when explanted *in vitro*. The role of the gland capsule was investigated following its removal, and development was found to be greatly reduced in its absence. The finding that both capsular and non-capsular mesenchyme prevented epithelial cell spreading in culture has significance in salivary gland organ culture since it tends to preserve the structural organisation of the epithelial component.

Using heat-treated and dry-ice treated capsular mesenchyme, epithelial cell spreading was inhibited and there was no progression of normal development suggesting regulatory factors, possibly labile and diffusible, were being produced by either component. Whether the live capsule exerted a specific organising action or simply

provided the mechanical conditions for the expression of developmental potential was not determined. But the experiments were valuable since they stimulated interest in organ culture and by establishing conditions for in vitro culture opened the way for studying interactive factors in salivary gland development.

Grobstein (1953a) repeated the work of Borghese (1950 a,b) using mouse submandibular salivary gland and again showed the importance of the capsule in permitting continued development. By using enzymatic separation techniques (trypsinisation), Grobstein showed that salivary gland epithelium was dependent upon its mesenchymal association for continued development. The heat-treated mesenchyme was unable to support epithelial growth showing that its action was not of a template nature (Grobstein, 1953 a,b).

By using other sources of mesenchyme from the same animal, characteristic submandibular salivary gland development occurred only if submandibular salivary gland cells were recombined with salivary gland mesenchyme - an absolute specificity not found in other organs whose epithelium would differentiate when combined with mesenchyme from other organs such as pancreas or kidney. Not only was the mesenchyme found to contain "information" about development, but the epithelium is also programmed in advance of the interaction for mesenchyme specificity. Thus, pancreatic epithelium with salivary gland mesenchyme differentiates as pancreas ; whereas salivary gland epithelium with salivary mesenchyme differentiates as salivary glands but not if pancreatic mesenchyme is used. Grobstein (1967 a,b) showed that mouse parotid gland mesenchyme could also evoke a developmental response from mouse submandibular gland epithelium.

If salivary epithelium was combined with its own mesenchyme in the presence of mesenchyme from another site, e.g. mammary gland (Kratochwil, 1969) then development was strongly inhibited. Various models can be proposed to

explain this mutual interference of the organising influence of mammary and salivary mesenchyme.

Experiments using species other than the mouse revealed that chick submandibular mesenchyme could evoke development of mouse submandibular epithelium (Sherman, 1960). Ball (1974 a,b), working with embryonic rats, found that the epithelial component of the submandibular gland would undergo limited development when incubated with embryonic bronchial mesenchyme. Ball also demonstrated differences between the major salivary glands in the rat. Whereas in the mouse, the requirement for submandibular mesenchyme is exacting, it is less so in the rat since bronchial mesenchyme will permit limited development of submandibular epithelium. The submandibular and sublingual glands differ for the sublingual gland attains greater complexity and thus has a less stringent requirement for homotypic mesenchyme. The sublingual gland will also undergo limited development with pancreatic and jaw mesenchyme both of which fail to induce further development of submandibular epithelium.

The parotid gland in the rat differs from the submandibular and sublingual gland in that its development is primarily post-natal (Schneyer and Hall, 1969, 1970).

Rat parotid epithelium is not as stringent as submandibular epithelium in its mesenchymal requirements. Lung, stomach and pancreatic mesenchyme (separated enzymatically) will support parotid salivary gland development but only to a limited degree and always less than that achieved with the own mesenchyme (Lawson, 1970, 1972). Morphogenesis proceeded comparably when either rat parotid or rat submandibular epithelium was combined with its own or the other glands mesenchyme. The *in vivo* production of amylase was maintained *in vitro* and was greatest when combined with submandibular mesenchyme.

Such studies demonstrate gross epithelial-mesenchymal interactions and specificities. Using essentially similar techniques, working with foetal mice, further studies have elucidated functional aspects of submandibular gland morphogenesis.

1.3 FUNCTIONAL DIFFERENTIATION

In the mouse, both the submandibular and parotid salivary glands have amylase activity. Amylase in the former gland arises in ductal epithelial cells and in the parotid from acinar cells (Junqueira and de Moraes, 1965 ; Rutter and Weber, 1965). In the rat, only the parotid salivary gland has significant amylase activity, that of the submandibular salivary gland remains at the foetal level (Lawson, 1970). This fact, combined with the predominantly post-natal development of the rat parotid gland enabled Lawson (1970, 1972) to investigate the relationship between epithelial-mesenchymal interactions and functional differentiation using amylase activity as a marker.

Reciprocation of mesenchymal activity was noted between the parotid and the submandibular salivary glands, but amylase activity was developed only by parotid epithelium whether with its own or with submandibular mesenchyme. This demonstrates that not only does the epithelium retain amylase specificity, but also that amylase induction requires mesenchyme. The mesenchyme thus contains information essential for expression of normal salivary gland epithelial properties.

Functional differentiation in vivo of rat submandibular salivary gland is thought to arise from cells of the terminal tubules (Jacoby and Leeson, 1959). The terminal tubule, or immature secretory unit, of rat submandibular gland persists for six weeks post-natally. Shortly before birth, proacinar cells (Yamashina and Barka, 1972) also termed cells with polymorphic granules (Dvorak, 1969) - differentiate from the terminal tubules.

Strum (1971) has shown peroxidase to be a marker for differentiation of proacinar cells believed to be direct precursors of acinar cells. Rufo and Barka (1975) used the histochemical localisation of peroxidase in their *in vitro* study of cell differentiation in the terminal tubule of foetal rat submandibular gland. As morphogenesis proceeded *in vitro* marked by the typical glandular branching pattern, the proacinar cells differentiated to form secretory acinar units in an analogous manner to that which occurs *in vivo*.

In 1970, Yohro concluded that neural influences were important in the post-natal functional development of mouse salivary gland secretory units. Close association between nerve axons and salivary epithelial cells is known *in vitro* (Friedmann and Hodges, 1975). However, differentiation proceeds normally *in vitro* even in the absence of parasympathetic neural innervation (Coughlin, 1975 a, b).

1.4 THE MOLECULAR BASIS FOR SALIVARY GLAND DEVELOPMENT

The relatively close approximation of the salivary gland epithelial and mesenchymal cells necessary for morphogenesis, for example, in transfilter experiments, indicates that induction is a short range phenomenon (Grobstein, 1956 a, b). Grobstein (1962) has also shown that heat treating either cell type results in failure of morphogenesis. The inductive process may therefore depend not only upon proximity but also upon cellular integrity.

The effect of mesenchyme on epithelium may be via a number of factors. Fell and Grobstein (1968), Golosow and Grobstein (1962) and Rutter, Wessells and Grobstein (1964) working on mouse salivary gland and pancreas alluded to the molecular action of mesenchyme in morphogenesis. Pancreatic epithelium failed to develop either if salivary mesenchyme was absent or if the growth medium had only a low concentration of cell free embryo extract. Pancreatic epithelium will differentiate either if salivary mesenchyme is present or if a high

concentration of embryo extract is present in the medium. When salivary epithelium replaces pancreatic epithelium, only salivary mesenchyme induces morphogenesis, the concentration of embryo extract alone or in combination with the mesenchyme having no effect on differentiation. Salivary mesenchyme therefore provides sufficient factors necessary for pancreatic epithelium to develop but also provides an additional factor or factors necessary for salivary epithelial development. Cell-cell contact as a determinant was ruled out by the studies of Grobstein (1964).

The role of collagen as a factor in salivary gland development was studied by Kallman and Grobstein (1965 ; 1966). The first of these papers reported an autoradiographic study using tritiated proline. Labelling of collagen occurred only if the salivary mesenchyme was subjected to a pulse of tritiated proline but not if the pancreatic epithelium was pulsed. The conclusion was that collagen was being synthesised by the mesenchyme and secreted into the interspace in soluble form. There, active polymerisation occurred in relation to the epithelial surface. Shortly afterwards, Grobstein and Cohen (1965) using mouse salivary epithelium and mesenchyme showed that collagenase but not hyaluronidase altered the pattern of salivary gland development.

Collagen, whilst being one factor of relevance to epithelial morphogenesis is probably not specifically responsible for the salivary gland epithelial-mesenchymal interaction. Tropocollagen does not replace salivary mesenchyme for though non-salivary mesenchyme labelled with tritiated amino acids transfers large molecular weight labels to the epithelial surface, morphogenesis does not follow. Collagen itself therefore was involved in development but it alone was not sufficient to support epithelial morphogenesis.

The identity of molecules which control specificity, inductive capacity and growth of mesenchymal and epithelial cells is still largely unsolved.

1.5 THE EPITHELIAL-MESENCHYMAL INTERFACE

Previous studies on the molecular basis of morphogenesis focussed attention on the epithelial-mesenchymal interface. Grobstein and Cohen (1965) postulated the role of an epithelial surface mucopolysaccharide in morphogenesis particularly in relation to collagen. Wessells and Cohen (1968) using collagenase in a variety of tissues including mouse pancreas, showed that in addition to removing collagen, the enzyme, at the ultrastructural level, caused disruption of the basal lamina and dissolution of the extracellular matrix adjacent to the basal lamina.

The observation of marked heterogeneity of collagenase preparations renders difficult the absolute identification of crucial structures in these developmental interactions (Dow, Harding and Powell, 1981).

Bernfield and Banerjee (1971) studied the epithelial-mesenchymal interface by an autoradiographic technique. The epithelial surface mucopolysaccharide postulated by Grobstein and Cohen (1965) was shown to be an acid mucopolysaccharide. Morphogenesis was visualised to be the result of cleft formation in the epithelium, followed by the formation of acid mucopolysaccharide-protein complexes between the cell layers which in turn initiated collagen synthesis to stabilise the new morphology.

Evidence for this theory was provided by Bernfield, Banerjee and Cohn (1972) who, using a two-stage enzymatic treatment of mouse submandibular salivary glands, showed that the acid mucopolysaccharide was localised within the epithelial basal lamina and that morphogenesis depended on its presence ; loss of this material arrested normal morphogenesis which was restored on its synthesis which was greatest at the sites of incipient branching.

Cleft formation is essential and occurs at a very early stage in salivary gland morphogenesis, seeming to determine the pattern of later events.

The role of cytoplasmic microfilaments and microtubules in salivary gland development became apparent (Spooner and Wessells, 1970 ; Spooner, Yamada and Wessells, 1971 ; Wessells, Spooner, Ash, Bradley, Luduena, Taylor, Wrenn and Yamada, 1971 ; Spooner and Wessells, 1972). By the use of cytochalasin B, which disrupts microfilaments reversibly without visibly altering cytoplasmic or spindle microtubules (Carter, 1967), and colchicine which disrupts microtubules (Robbins and Gonatas, 1964), the role of both was elucidated.

Each drug given individually inhibits morphogenesis. Colchicine does not cause loss of clefts present at the time of drug application. Colchicine given during the recovery period from cytochalasin allows cleft formation demonstrating that clefts can form in the absence of microtubules and in the absence of mitosis. The implication is that microfilaments, probably via contractile activity, are involved in salivary gland morphogenesis. Older clefts were not sensitive to cytochalasin pointing to other factors being involved in the stabilisation of salivary tissue. Salivary mesenchyme may act to alter microfilament function since salivary epithelium in the presence of bronchial mesenchyme still possesses cytoplasmic microfilaments though development does not occur. The time scale of such salivary epithelial-mesenchymal interaction of several hours perhaps extending for days (Grobstein, 1968) indicated that the interaction was not an instantaneous event but a complex extended process (Gossens and Unsworth, 1972).

1.6 CELL TO CELL CONTACTS IN SALIVARY GLAND DEVELOPMENT

Studies on epithelial-mesenchymal interaction (Grobstein and Dalton, 1957 ; Auerbach, 1960) have involved the separation of the two components at some early developmental stage by a filter technique. Even when the cell contact is prevented, morphogenesis may still occur suggesting diffusible factors to be of importance. Grobstein (1959) using autoradiographic techniques showed that labelled proline

incorporated into salivary mesenchyme is transferred across the filter and bound extracellularly on the epithelial side in a form removable by collagenase (Kallman and Grobstein, 1965 ; Bernfield, 1970).

In other studies direct epithelial-mesenchyme contact is produced and the interaction studied in this way may clearly involve cell to cell contacts (Borghese, 1950 a, b ; Lawson, 1972). Borghese (1950 a) noted the presence of the parasympathetic submandibular ganglion in salivary gland explants ; thus epithelial-mesenchymal and epithelial-nerve contacts may both be of importance. Coughlin (1975 a, b), working on embryonic mouse submandibular salivary gland, showed that in vivo epithelial-axonal contacts were not seen but they did occur in vitro. In addition, in vitro axonal outgrowth paralleled both in time and space early cleft formation and epithelial morphogenesis. In a later paper Coughlin and Rathbone (1977) raised a dichotomy of evidence by showing that morphogenesis proceeds normally even in the absence of the neuronal element.

Cutler (1977) compared the development of rat submandibular salivary glands in vitro with previous results obtained in vivo (Cutler and Chaudhry, 1973 a, b, c). He found no evidence of epithelial-nerve contacts during morphogenesis in vitro but epithelial-mesenchymal contacts were present and appeared after the morphogenetic branching pattern was established.

On this basis, the theory was proposed that these epithelial-mesenchymal contacts, which took the form of approximated cell membranes separated by a 15 nm gap containing amorphous material, were perhaps involved in differentiation and continued growth of the gland after the initial branching pattern was established.

1.7 CONCLUSIONS

The molecular aspects of salivary gland growth and development as elucidated from in vitro techniques provide

a basis from which single cell cultures and the effect thereon of neurotransmitters can be studied in an attempt to clarify the neuronal role.

CHAPTER 2

CELLULAR STRUCTURE OF MURINE AND HUMAN SALIVARY GLANDS

2.1 LOCATION OF MURINE SALIVARY GLANDS

Salivary glands belong to two main groups, major and minor. The major salivary glands are relatively large symmetrically paired structures referred to as the parotid, submandibular and sublingual salivary glands. In Figure 1 these glands are shown in an adult CFLP mouse following a midline incision to expose the neck region.

The submandibular salivary glands are well encapsulated, are the larger major salivary glands in mice and lie either side of the midline extending from the undersurface of the mandible anteriorly to the clavicle posteriorly. The normally darker-looking sublingual glands are on their anterolateral surface, separated by the respective capsules. This relationship is seen in a newborn animal in Figure 2. Both pairs of glands have initially separate excretory ducts running between the mylohyoid muscle and oral mucosa anteriorly to open behind the lower incisors.

The parotid salivary glands are more diffuse, lying further laterally. They are less well capsulated and may be interspersed with adipose tissue. In the newborn animal they lie subcutaneously along the outer border of the cheek opposite the ramus of the mandible (Figure 3). In adult animals the glands may extend as far posteriorly as the clavicle and may overlap the submandibular salivary glands medially. The parotid excretory duct is subcutaneous and initially runs anteriorly but then, as in humans, turns medially piercing the buccinator muscle to open opposite the molar teeth.

Minor salivary glands in mice are scattered throughout the mouth in an inconstant pattern. Typically, however, in

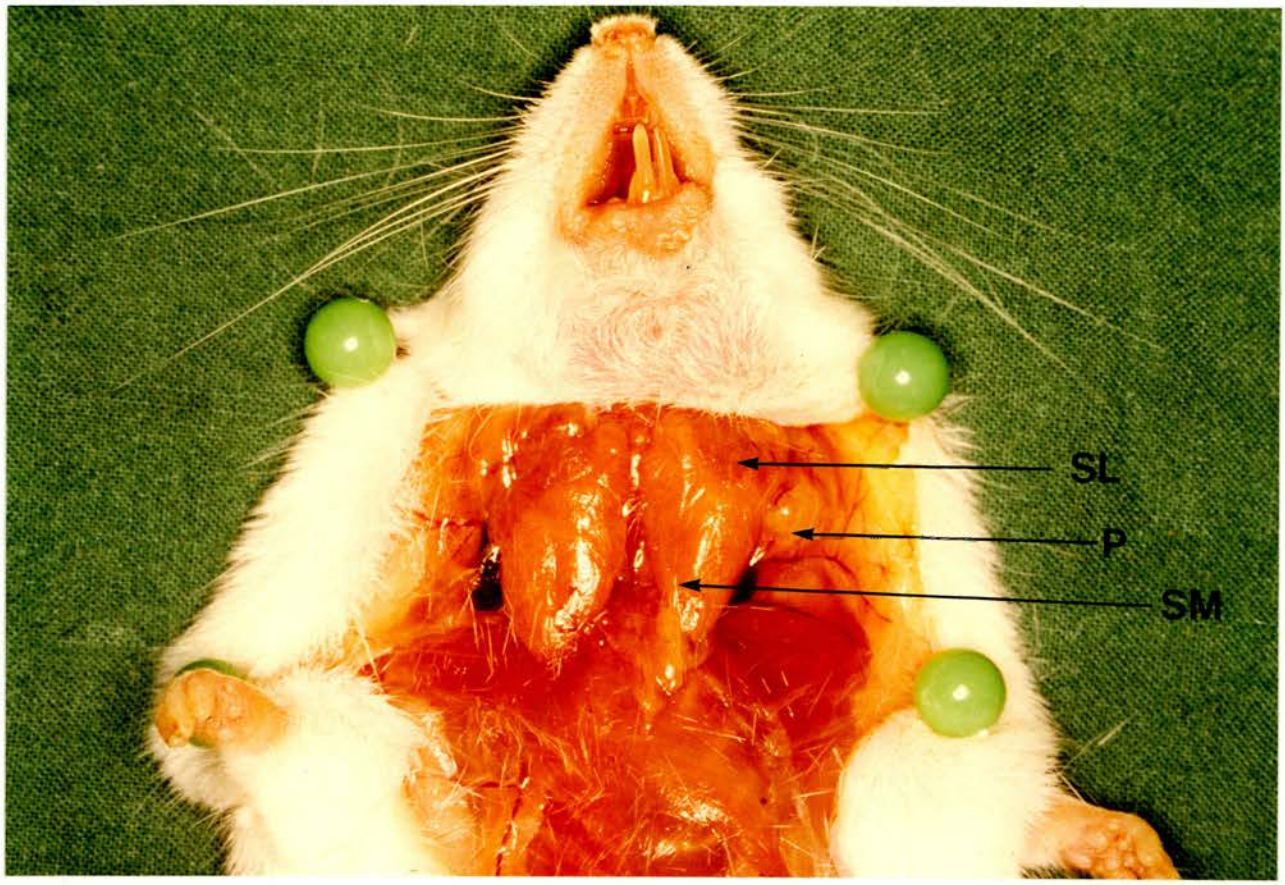


Figure 1

The neck region of an adult CFLP mouse. The large submandibular salivary glands (SM) lie on either side of the midline and have the darker sublingual (SL) salivary glands on their anterolateral aspect. The parotid (P) salivary glands lie further laterally but may extend towards the midline.

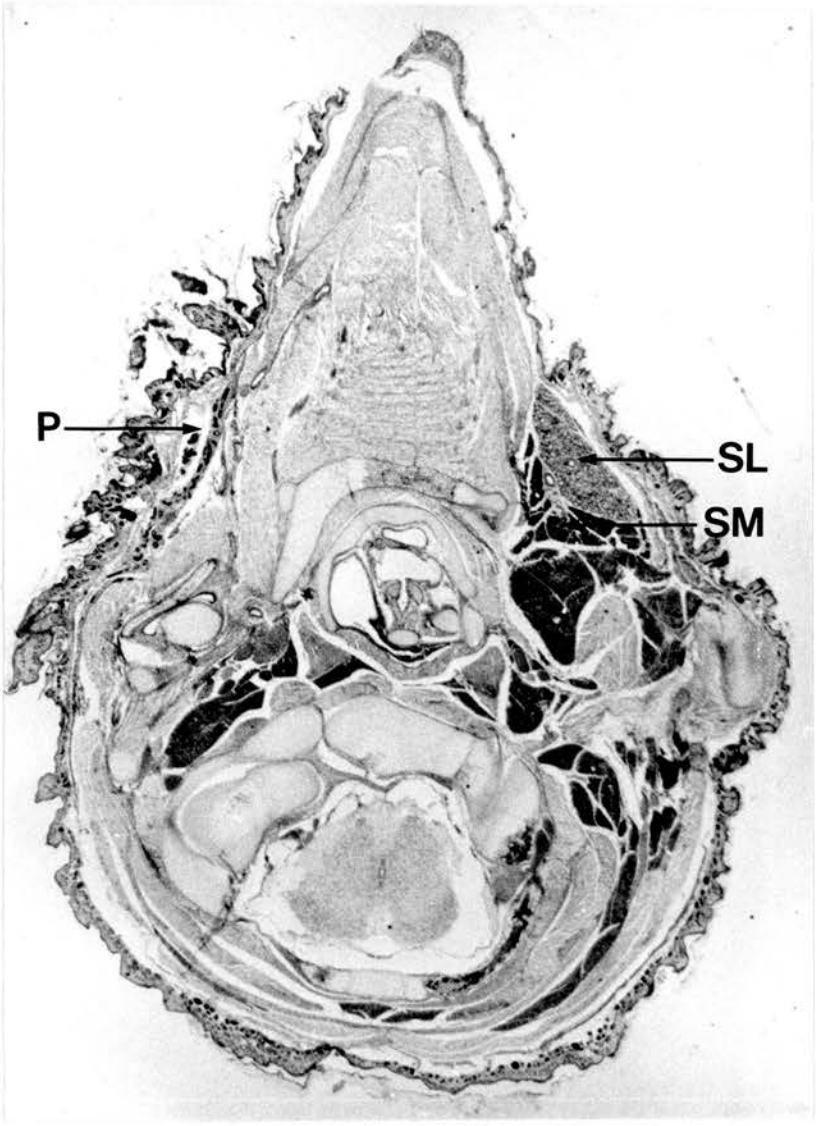


Figure 2

Horizontal section through the neck and floor of mouth region of a newborn (two week old) CFLP mouse. The parotid (P) sublingual (SL) and submandibular (SM) glands can be seen. Stained haematoxylin and eosin the paler mucous acini in the sublingual gland and darker serous acini of the submandibular gland can be discerned.

x 10

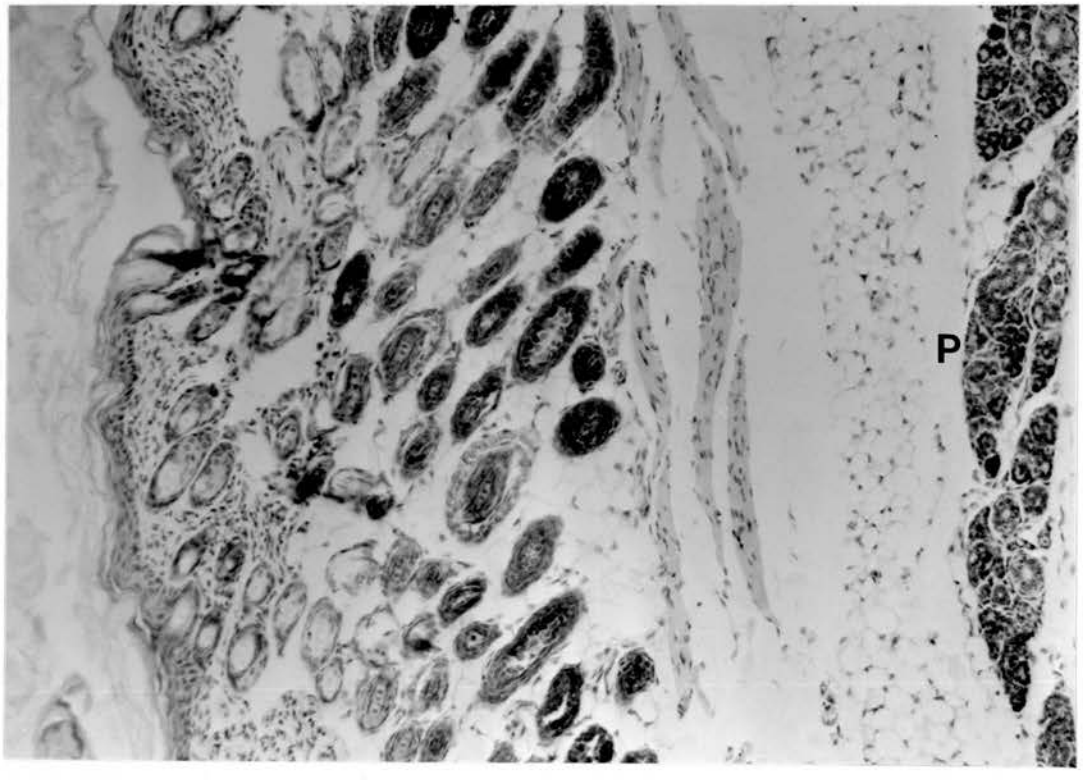


Figure 3

Oblique section stained haematoxylin and eosin through the lateral neck region of a newborn (two week old) CFLP mouse. The subcutaneous position of a parotid (P) salivary gland is seen.

x 100

humans they are found in the mucosa and underlying tissues of the palate, faucial region, the lips, the cheek, the tongue and the floor of the mouth. The minor glands which are found in the lip can easily be demonstrated following eversion of the lip (Figure 4).

2.2 NORMAL DEVELOPMENT AND STRUCTURE OF MURINE MAJOR SALIVARY GLANDS AND HUMAN MINOR SALIVARY GLANDS

2.2.1 Murine Major Salivary Gland Development

The development of the major salivary glands follows a similar pattern in all glands. Histological studies such as that of Borghese (1950 a) concentrated on the development of the submandibular salivary glands and it is these glands which are reported below.

The glandular primordia stem from proliferating buds of oral epithelial cells, at about the twelfth day of gestation. Although Borghese considered the gland ectodermal in origin in mice, in humans its relationship to the buccopharyngeal membrane is less clear and they have been considered of endodermal origin (Hamilton, Boyd and Mossman, 1962).

The downgrowing epithelial bud may form a groove along the floor of the mouth and may develop at the site of the future gland in the adult mouse. It has been postulated that the initial epithelial cell downgrowth occurs at the site of the future submandibular salivary gland but that the major portion of the excretory duct arises by an invagination of the floor of the mouth. This invagination may or may not include the excretory ducts of the sublingual salivary glands.

By day 14 in the mouse the epithelial bud has become branched and the future capsular component derived from the surrounding mesenchymal tissue is closely approximated. At day 15 the hollowing of the initial solid epithelial buds occurs forming a simple central lumen within each. The acini are not yet functioning to produce a secretion.

Figure 4

Eversion of lower lip to demonstrate the finely nodular epithelium below which lie the numerous minor labial salivary glands.

Although the initial epithelial down growth is undifferentiated the various glandular components develop from these cells. Acini develop from proacinar cells identifiable histochemically by their peroxidase reaction (Dvorak, 1969). Development of the submandibular salivary gland of the rat from a solid tube of cells through a branching tubulo-acinar structure is continued post-natally (Jacoby and Leeson, 1959). Branching of the terminal tubules produces further acinar differentiation with the terminal tubules being linked to intralobular striated ducts via first order (and later following a decrease in size to second order) intercalated ducts. It is the intercalated ducts which by producing secretory granules become the granular convoluted tubules.

2.2.2 Identification of mesenchyme

A specific salivary mesenchymal cell has not been identified. The term mesenchyme describes connective tissue cells, blood vessels and nerves. A specific role for blood vessels in salivary gland development has not been determined and the fact that normal development may proceed *in vitro* could argue against one being found. Blood vessels may survive in salivary gland explants and lie on either side of the largest ducts as in the normal gland (Borghese, 1950 a). Nerves are thought also not to be of prime importance in development since Coughlin (1975 a) could detect no effect on mouse submandibular gland development *in vitro* following removal of the sublingual ganglion. Cutler and Chaudhry (1973 c) reported that in the rat submandibular gland *in vivo* epithelial nerve contacts were present but appeared after the developmental branching pattern of the gland was established. These epithelial-nerve contacts were not found *in vitro* (Cutler, 1977).

The mesenchyme which closely invests the epithelial bud during development is considered to be composed of fibroblasts on morphological grounds (Borghese, 1950 a) and because of the ability to produce collagen (Grobstein

and Cohen, 1965). Early experiments (Borghese, 1950 b) show that on an area basis the mesenchymal component is several times larger than the rudimentary epithelial element. This relationship is relatively reduced as development proceeds and it is known that this early mass effect is necessary in determining the range of inductive activity (Koch and Grobstein, 1963). Within the mesenchymal tissue, which is identified as all the non-epithelial primitive ductal tissue, fibroblasts are arranged in a characteristic pattern (Borghese 1950 a, b) in vitro. The capsule of connective tissue is composed of concentric lines of fibroblasts which later form the gland septae.

Attempts have been made to identify substances produced by mesenchymal cells which will support normal gland development. No one factor has so far been isolated and the process of development is likely to be more complex. In addition regional differences in fibroblasts from in vitro recombination experiments and in vivo transplantation experiments (Billingham amd Silvers, 1967) are known to exist. The inductive effects of mesenchyme may in part depend on the epithelial cell cycle. Pancreatic epithelial cells require to undergo division of the terminal tubule cells for differentiation to follow (Wessells and Wilt, 1965). An epithelial cell so induced may then be able to involve adjacent epithelial cells via communication at specialised cell junctions.

Finally, mesenchymal cells are important not only in the inductive phase of acinar and ductal development but in the maintainance of epithelial specificities (Billingham and Silvers, 1963). The salivary gland septae and capsule formed during development probably are a manifestation of a requirement for this maintenance effect. Not all salivary glands have a gland capsule. Minor salivary glands lack a capsule and it is these glands which are least specialised in terms of the production of enzyme and protein products (Eversole, 1972 ; Harrison, 1974). It could be coincidence

that in humans these minor salivary glands are those in which malignant tumours have been stated to be most frequent (Ranger, Thackray and Lucas, 1956).

2.2.3 Human Minor Salivary Gland Development

A number of authors have studied the morphological development of human labial salivary glands (Bromann, 1927 ; Fischel, 1929 ; Zimmerman and Zeidenstein, 1951 ; Arey, 1965 ; Goodman and Stern, 1972). The latter authors performed the largest study examining 80 foetuses. The process of development is similar to that for major salivary glands with an initial downgrowing epithelial bud being formed at 65 days gestation (Figure 5). The bud is of one cell type and following cavitation the resulting lamina contains the two cell layers (Figure 6). Goodman and Stern propose that the process of cavitation proceeds by cellular rearrangement (Patten, 1958) rather than by ischaemic necrosis (Glucksmann, 1951). Histologically the absence of cell debris and macrophages would seem to support this view. Repeated bud branching produces numerous acini but most glands have a single excretory duct. By 180 days the glands are histologically mature.

Unlike murine major salivary gland acini which are non-functional at birth there is evidence that human labial salivary glands are functional in utero. The presence in these ducts of eosinophilic material staining like mucus suggests active acinar secretion although the duct cells could conceivably be producing this.

2.2.4 Structure of Murine Salivary Gland Components

The accepted classification of the components of salivary glands is that proposed by Tupa (1926). The nomenclature used originally referred to the submandibular salivary gland of the mouse and rat but has since been applied to other glands in various species. The classification recognises five parenchymal components : the sero-mucous acini, the intercalated ducts, granular (or serous) tubules, the

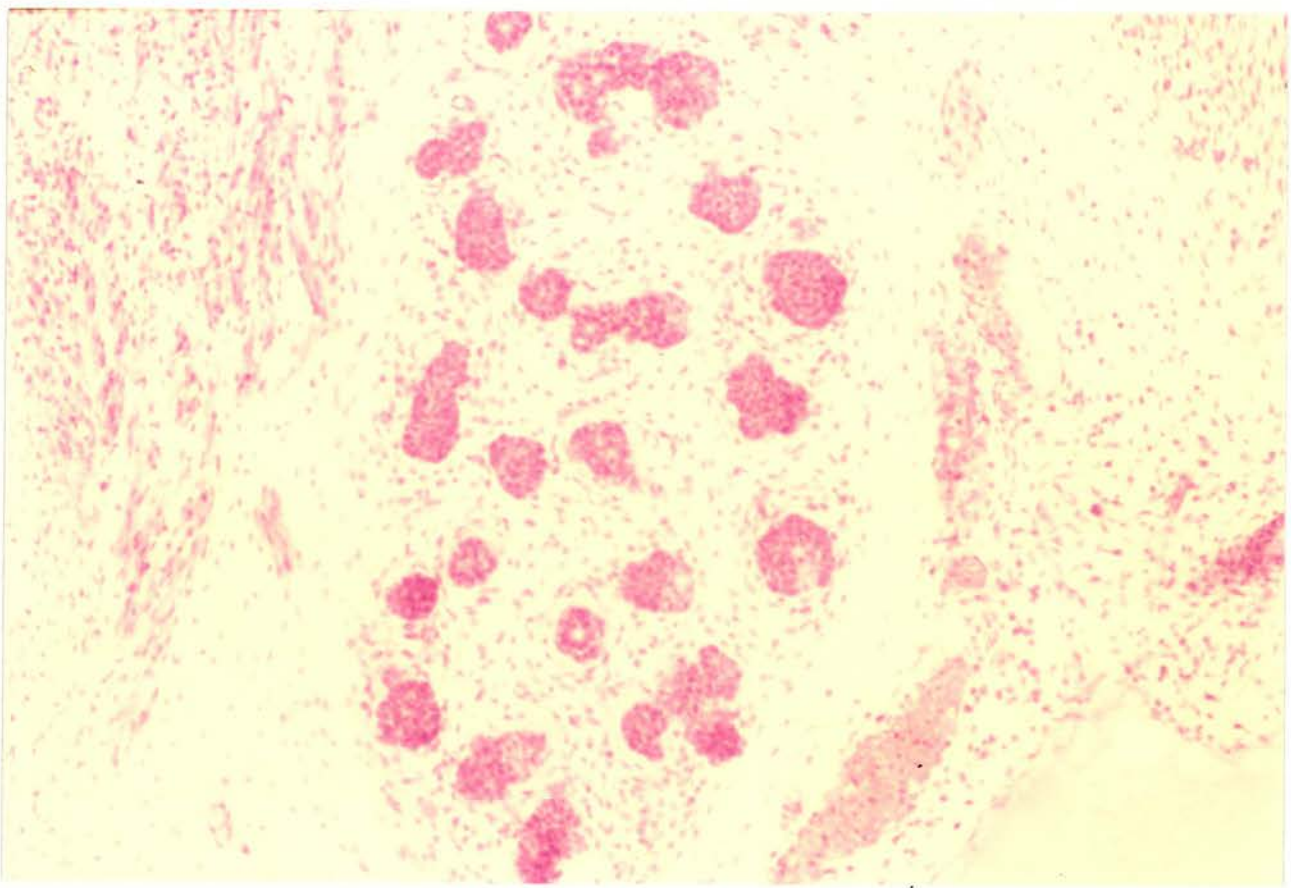


Figure 5

Developing bud of the human submandibular salivary gland around day 65 of gestation. The solid nature of downgrowing epithelium is apparent. Stained haematoxylin and eosin.

x 60

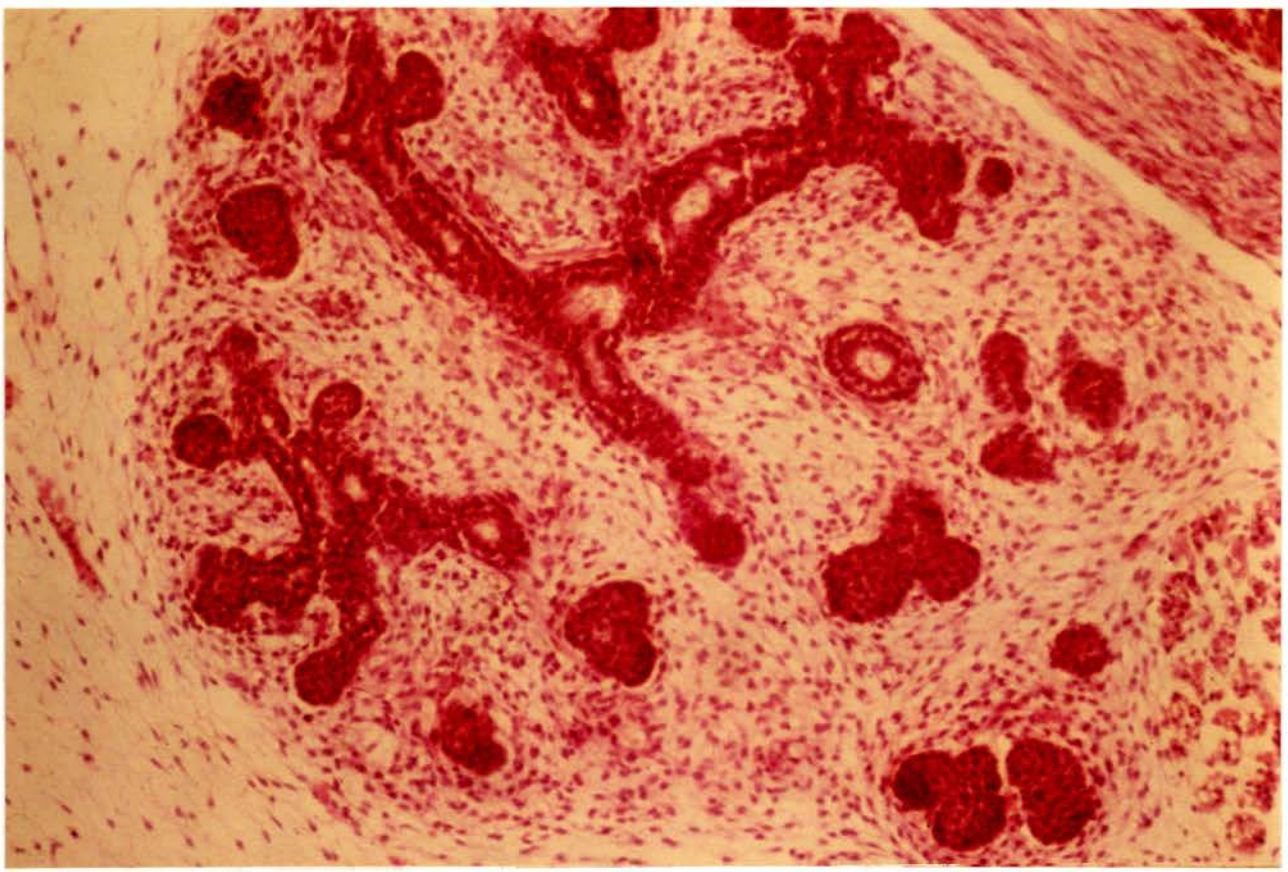


Figure 6

Developing bud of the human submandibular salivary gland around day 90 of gestation. The initial solid bud of oral epithelium has now acquired a central lumen. Stained haematoxylin and eosin.

x 40

striated ducts and the excretory ducts. Some components such as the granular tubules are hormonally dependent and their proportion varies with the sex of the animal. The overall arrangement of the components of the submandibular salivary gland is illustrated in Figure 7.

a) Acinar cells

Acinar cells in the mouse submandibular salivary gland undergo considerable pre-natal change. Firstly, differentiation from terminal tubules rapidly occurs ; secondly, gland secretory activity begins. Proacinar cells, intermediate between acinar and terminal tubule cells, characteristically have polymorphic granules (Dvorak, 1969). Although the duct system is actively involved in the formation of saliva (Burgen and Seeman, 1958) it is from the acini that secretion probably originates.

In cross section the acinar cells of adult salivary glands are described as pyramidal in shape with a basal epithelial nucleus (Figure 8) and apical secretory granules (Martinez-Hernandez, Nakane and Pierce, 1972). Stains for neutral or acidic polysaccharides (Shackleford and Klapper, 1962) give the mouse submandibular acinar cells a characteristic basophilic colour. Although acini are grouped around a central lumen this is not readily seen at the light microscope level (Yohro, 1970).

b) Cells of the intercalated duct

These cells are low cuboidal in shape, have a centrally placed nucleus and, like the acinar cells, rest on a basement membrane. They may have myoepithelial cells approximated round their periphery (Figure 9). Yohro (1970) considered that intercalated ducts (Figure 7) were composed of granular and agranular cells ; the granular variant may constitute the remnant of the terminal tubule cells. Distinction between first and second order intercalated ducts is not possible histologically. The second order intercalated duct leading

The Main Epithelial Components of the Male Mouse Submandibular Gland

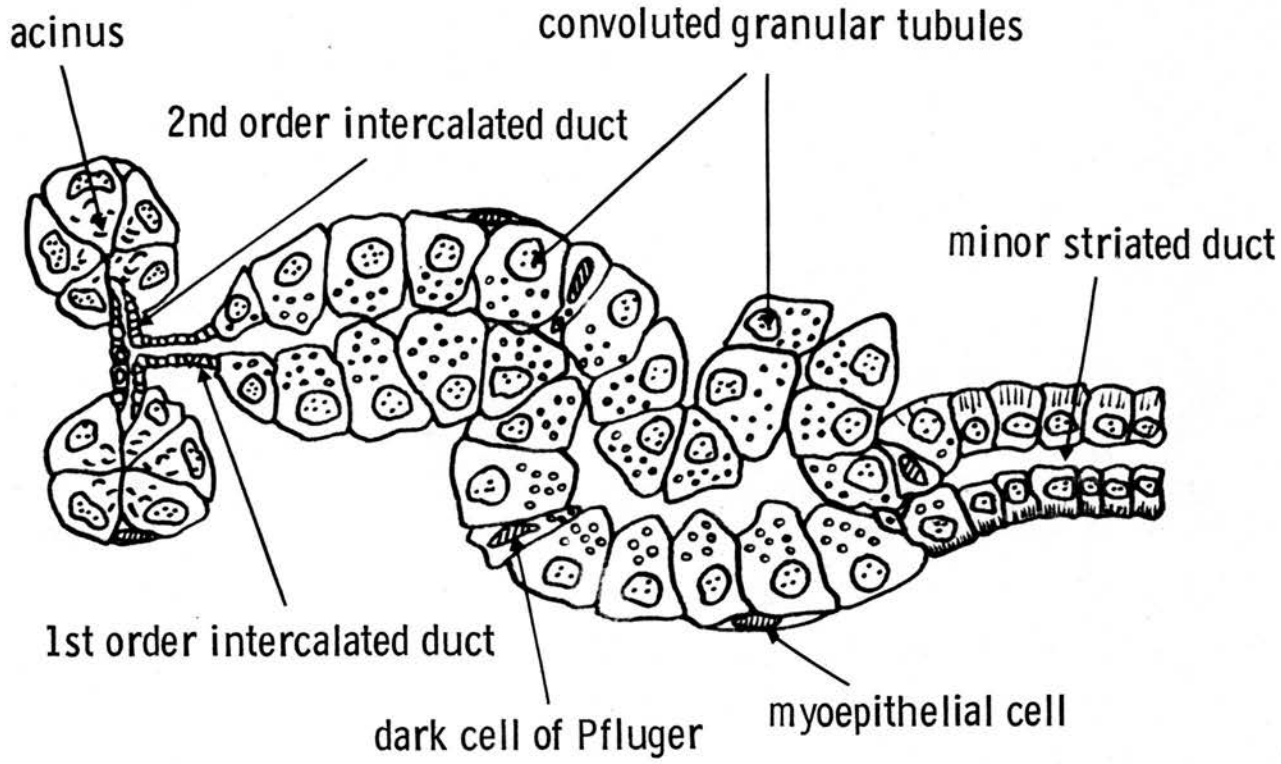


Figure 7

Diagrammatic representation of the cellular components of a murine submandibular salivary gland.

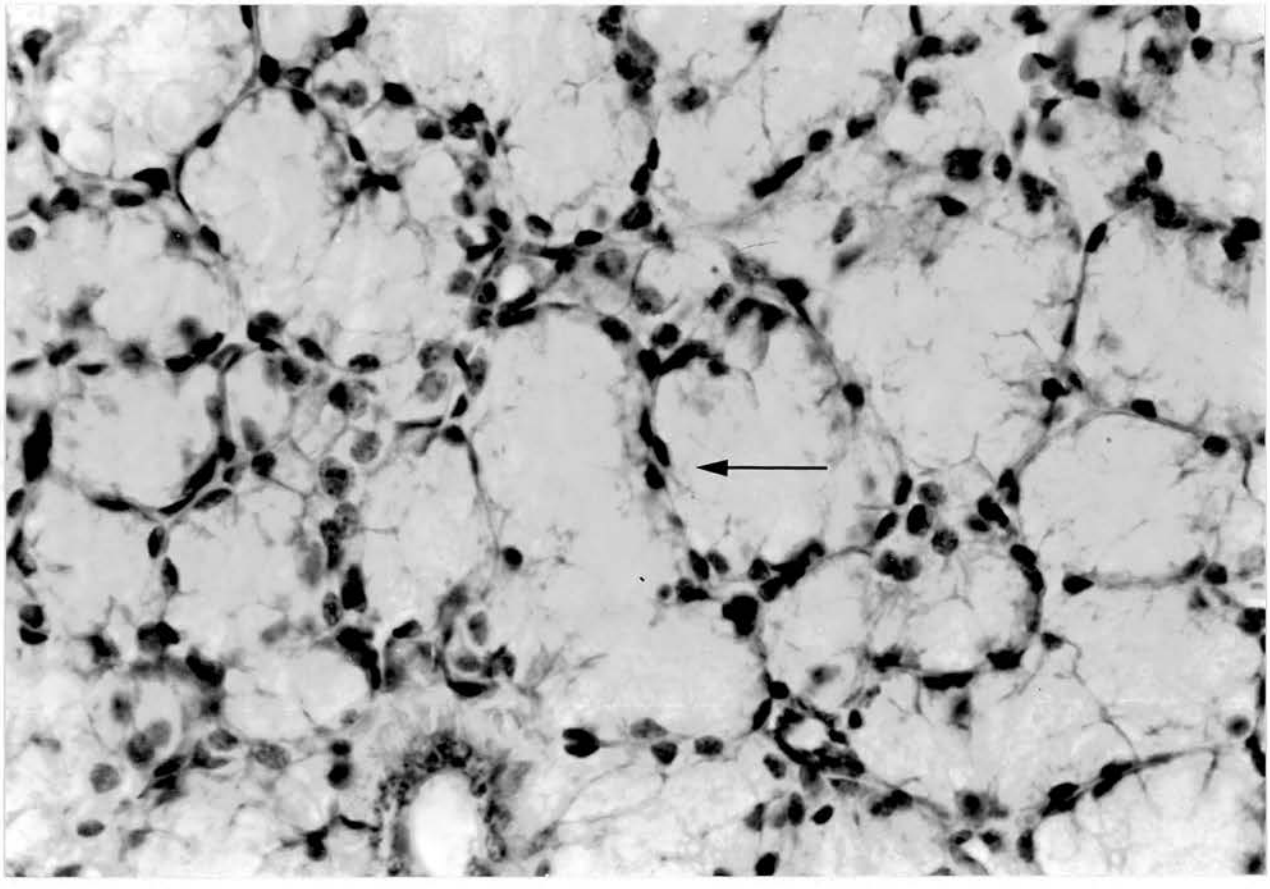


Figure 8

Murine submandibular salivary gland acinar cells with (arrow) pale cytoplasm, pyramidal shape and basally placed nuclei.

X 400

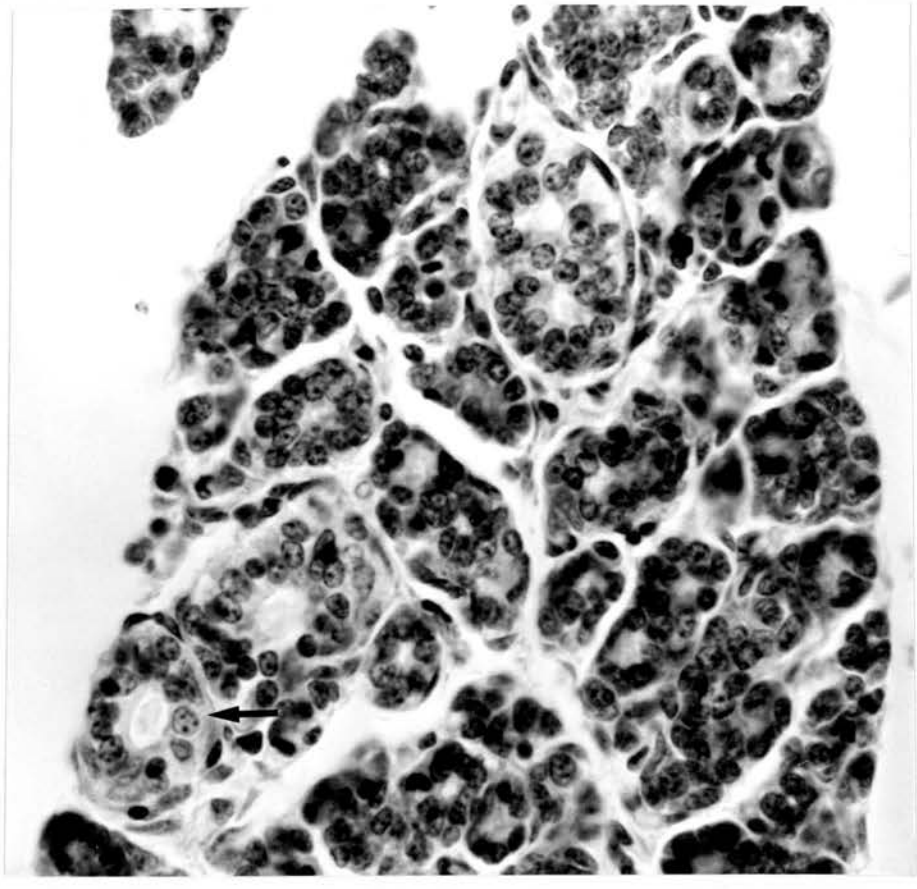


Figure 9

Intercalated duct cells in mouse submandibular gland. These cells are cuboidal in shape with a centrally placed nucleus (arrow). Stained with haematoxylin and eosin.

X 320

from the lumen of the acini is short (two or three cells) compared with the first order intercalated duct which links two or more acini and their second order ducts with the granular tubule cells.

c) Granular tubule cells

These cells are only present in the salivary glands of rodents and some other species (Aboubard, 1955). Most are wide columnar cells with large basal nuclei. The apical cytoplasm stains with toluidine blue. Tamarin and Sreebny (1965) considered there to be three cell types which represented sequential phases in a cyclic secretory process ; narrow agranular cells, light granular cells and wide dark granular cells. Rapid depletion of the granular tubule cell granule content follows starvation.

The marked morphological sexual dimorphism of the mouse submandibular salivary gland involves the granular tubule cells (Lacassagne, 1940 a, b, c ; Junqueira, Fajer, Rabinovitch and Frankenthal, 1949 ; Hosoi, Nakamura and Ueha,1977; Hosoi, Aoyama and Ueha, 1978). The differences between the sexes arises during postnatal development at the time when granular tubules are differentiating from the striated duct system (Chaulin-Serviniere, 1942) into which they lead (Figure 7).

d) Striated and excretory duct cells

Striated duct cells are present in most major salivary glands. The cells are low columnar in type, have centrally placed nuclei and rest on a basement membrane. In the basal cytoplasm perpendicular striations may be present. Striated duct cells probably have some functions similar to granular tubules and in species which lack granular tubules, a complex striated duct cell pattern is found (Shackleford and Wilborn, 1968, 1969, 1970 a, b).

The excretory ducts join to form the main excretory duct. A double-layered structure for the excretory duct is

characteristic. Duct cells have an important role in the alteration of electrolyte composition of saliva (Burgen and Seeman, 1958). More recently micro-puncture and perfusion studies (Young and Schogel, 1966 ; Young, Fromter, Schogel and Hamann, 1967) in rat submandibular salivary gland suggest a fluid secretory function for excretory ducts such that isotonic perfusion fluid is converted to a hypotonic end product.

e) Myoepithelial cells

These cells subserve a specialised contractile role in the spontaneous secretion of saliva (Emmelin, Garrett and Ohlin, 1968). Myoepithelial cells arise in association with the development of secretory cells by cyto-differentiation of epithelial stem cells in the distal buds and terminal tubules (Cutler and Chaudhry, 1973 a). They are not prominent normally but surround the basal surface of acini, intercalated ducts and granular tubules (Figure 10). They are present between the gland cells and the basement membrane. Atypical patterns of distribution do occur since, although they do not surround striated or excretory duct cells, they may be limited to one portion of the gland such as the intercalated duct region in rat parotid (Garrett and Parsons, 1973).

2.2.5 Structure of Human Minor Salivary Gland Components

Human labial salivary glands share some morphological features with murine major salivary glands but there are complexities in drawing more precise comparisons. For it must always be borne in mind that the functional state of the cell may alter its morphology, and that transitional changes between cell types may be observed.

a) Acinar cells

Unlike the mouse submandibular gland which may have mucous and serous-type acini, human labial glands are predominantly mucous with very few, if any, serous acini

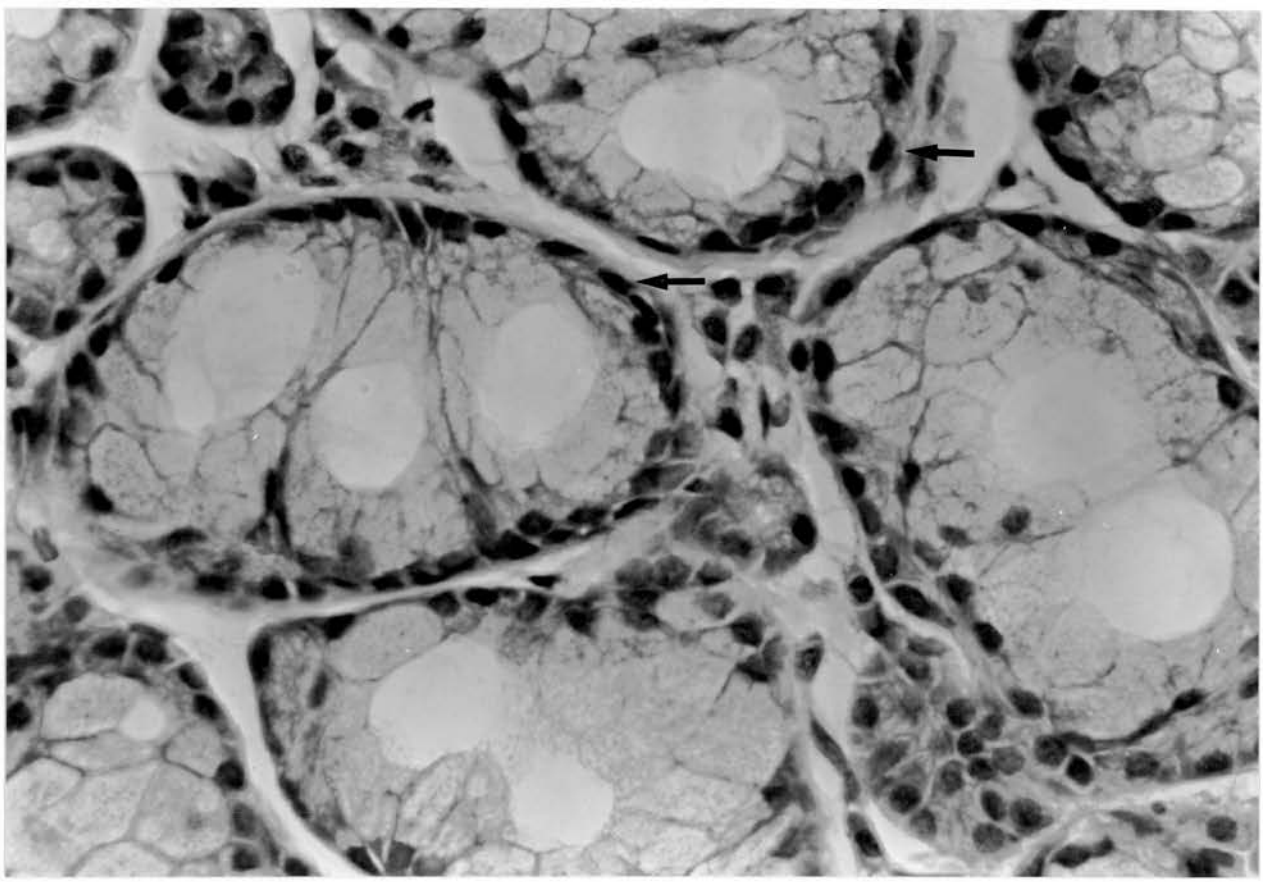


Figure 10

Site of myoepithelial cells (arrow) in mouse submandibular gland. These cells surround the basal surface of acini. Stained haematoxylin and eosin.

X 500

(Tandler, Denning, Mandel and Kutscher, 1969 a ; Provenza, 1964 ; Warwick, Bernard and Meskin, 1964). In contrast to the mouse submandibular salivary gland which is well encapsulated the human labial gland lacks a discrete capsule (Provenza, 1964).

b) Cells of the intercalated duct

The presence of these cells in human labial salivary glands is variable. When present, they resemble the murine cells in being of a low cuboidal form with a centrally placed nucleus and rest on a basement membrane (Figure 11). In their absence acini are continuous with the intralobular duct. In some units the transition from acinus to duct may be graded such that for part of its length the intercalated duct may consist of an admixture of duct cells and mucous cells of an acinar type (Tandler, Denning, Mandel and Kutscher, 1970). Approximately three acini usually drain into a single intercalated duct.

c) Granular tubule cells

These are not found in human salivary glands but are confined to the salivary glands of some orders of rodents, some bats and some insectivores (Aboubarb, 1955).

d) Striated duct and excretory duct cells

Conventionally (Tupa, 1926) striated duct cells lead on from the intercalated duct. In labial salivary glands, unlike human major salivary glands (Figure 12), the characteristic basal striations of striated duct cells are uncommon (Tandler, Denning, Mandel and Kutscher, 1970).

Excretory duct cells may have a double layered structure. When within the oral epithelium the cells are of stratified squamous type whereas they are pseudostratified (Figure 13) or tall columnar when within the lamina propria.

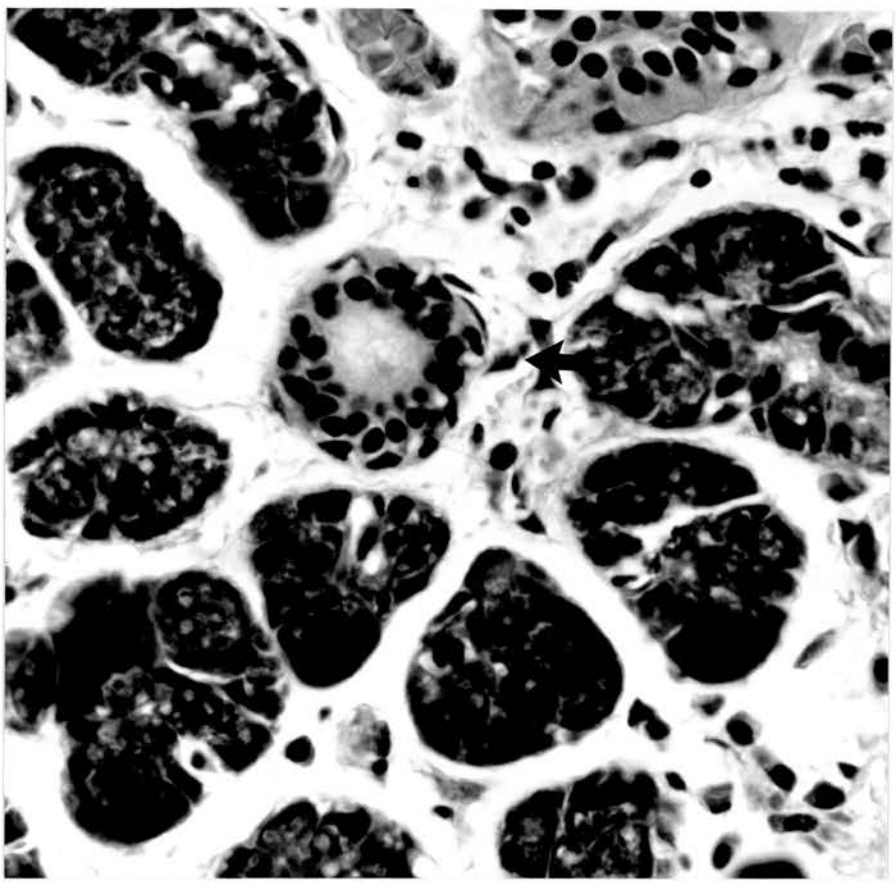


Figure 11

Intercalated duct cells of human labial salivary gland. These cells (arrow) are low cuboidal in type with a centrally placed nucleus. Their occurrence in these glands is variable. Stained haematoxylin and eosin.

X 400

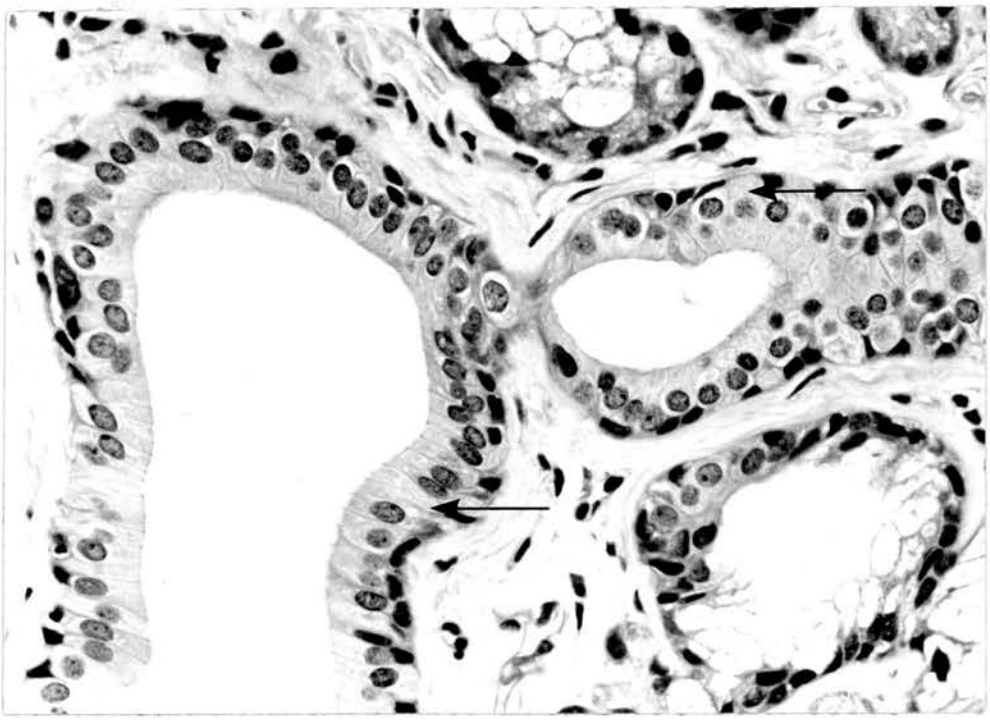


Figure 12

Striated duct cells from human labial salivary glands. Typical basal striations (arrow) are present but these are infrequent in human labial glands. Stained haematoxylin and eosin.

X 350

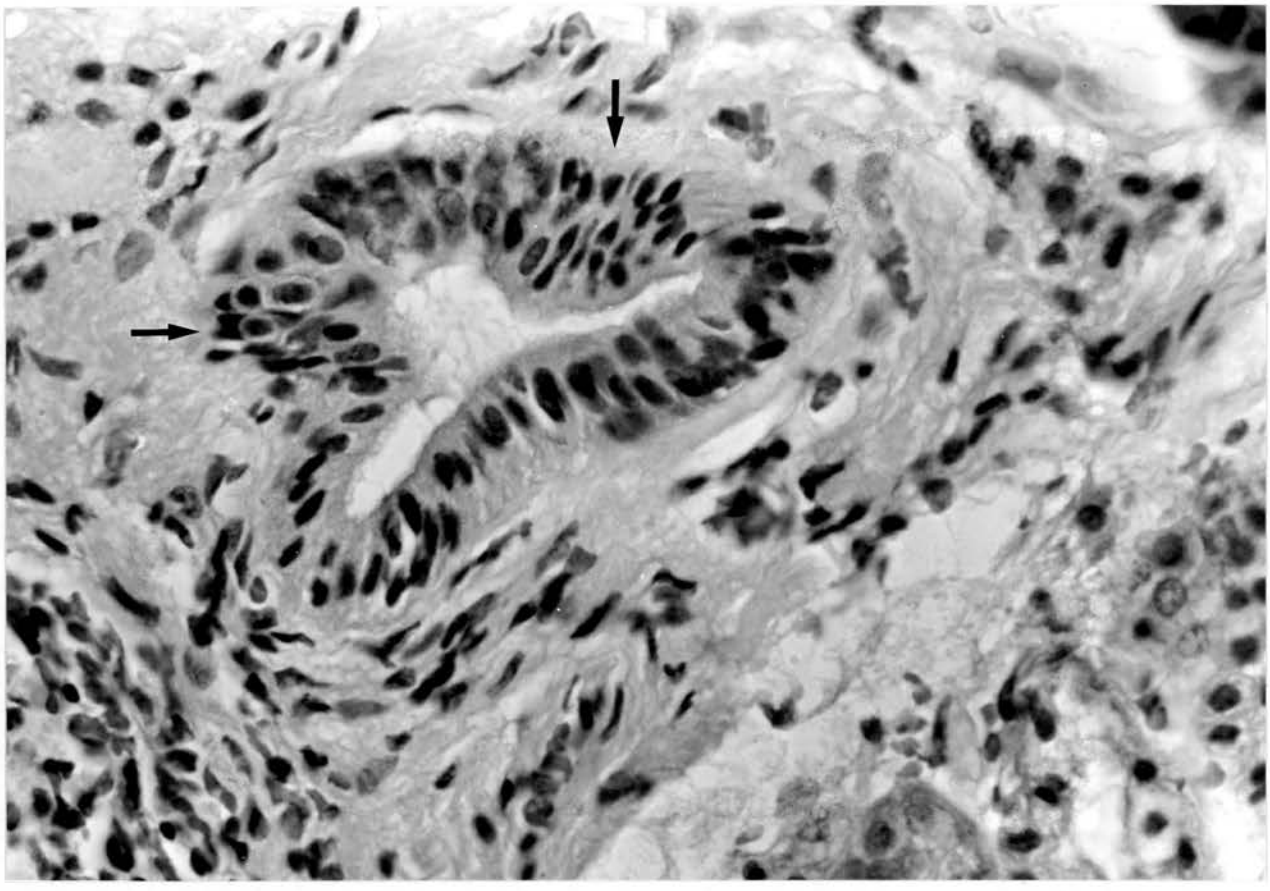


Figure 13

Excretory duct of human labial gland in the lamina propria. A pseudostratified appearance (arrow) is commonly found at this site. Stained haematoxylin and eosin.

X 460

2.2.6 Ultrastructure of Murine Major Salivary Glands

The main ultrastructural features of importance are cell to cell contacts, the presence and distribution of intracellular organelles and the relationship of salivary gland cells to autonomic nerves. Previous reviews of the ultrastructural features of mature and developing salivary glands of rodents cover these features in detail (Scott and Pease, 1959 ; Leeson and Jacoby, 1959 ; Tamarin and Sreebny, 1965 ; Line and Archer, 1972)

a) Acinar cells

Farquhar and Palade (1963) reviewed junctional complexes in various types of mammalian epithelia. Of the three types of cell junction zonula occludens, zonula adherens and macula adherens only the macula adherens (desmosome) was frequently found in human parotid salivary gland. In mouse and rat parotid acinar cells (Parks, 1961) desmosomes are numerous and found along most of the lateral cell membranes. Desmosomal junctions are also present at the apical borders with intercalated duct cells. The basal plasma membrane may form desmosomes with underlying myo-epithelial cells.

Intracellularly the position of the nucleus is governed by the number of secretion granules present (Martinez-Hernandez, Nakane and Pierce, 1972). During fasting, when secretion granules accumulate, the nucleus is displaced basally (Amsterdam, Ohad and Schramm, 1969). The prominent intracellular organelle is the rough endoplasmic reticulum indicative of an active secretory function. Yohro (1971) studied the relationship between nerve terminals and cellular junctions in young and adult mouse submandibular glands. The conclusion was that shortly after birth direct neuronal-acinar contacts were present but in the adult gland virtually no neuronal axonal or synaptic elements were found inside the basement membrane. The author states that the acinar cells may still be innervated since axonal expansions come into contact with acinar cells. There was no separation of neuronal elements into cholinergic or adrenergic components

in Yohro's study but in monkey submandibular salivary gland only cholinergic nerve endings are observed within acini (Kagayama, 1971).

b) Cells of the intercalated duct

The lateral and basal plasma membranes of intercalated duct cells have all three types of junctional complex present and such specialised cell contacts are numerous.

The nucleus is large (Leeson and Jacoby, 1959) and a moderate amount of granules may be found apically (Shackleford and Klapper, 1962 ; Shackleford and Wilborn, 1969). Caramia (1966) noted that the female mouse submandibular salivary gland was characterised by the presence of secretory granules in the intercalated duct nearest to the acinar cells.

Innervation of the intercalated duct cells is dense and direct (Yohro, 1971). The proposal was made that since developmentally the intercalated duct cells are derived from terminal tubules (Jacoby and Leeson, 1959 ; Yohro, 1970) then the intercalated duct innervation could be considered as a remnant of that of the terminal tubules.

c) Granular tubule cells

These cells exhibit some basal membrane infolding but lateral membranes are relatively smooth and desmosomes frequent. The cell nucleus is basally situated and both endoplasmic reticulum and mitochondria are numerous (Tamarin and Sreebny, 1965 ; Cutler and Chaudhry, 1973 c). The apical region contains secretion granules which have been distinguished into three types (Dorey and Bhoola, 1972). These granules were large and electron dense, large and moderately electron dense or small and moderately electron dense. A cell may contain more than one type of granule and has basal mitochondria similar to striated duct cells but also contains secretory granules in the apical cytoplasm (Caramia, 1966). The male murine submandibular gland has prominent secretory tubules

consisting of clear cells and the dark cell of Pfluger (Pfluger, 1866). The darker cell is comparable to the narrow granular cell described by Tamarin and Sreebny (1965) in the rat submandibular gland. The male granular tubule cell differs from the female cell in having intracellular canaliculi.

d) Striated duct cells

These cells are recognised by their prominent vertically-placed basal mitochondria between the infoldings in the plasma membrane. Lateral cell membranes in these columnar cells are also plicated and contain numerous desmosomes. The extensive system of plasma membrane infolding is thought to be related to electrolyte transport such as in renal cells (Berridge and Oschman, 1972). In species whose salivary glands lack granular ducts three types of striated ductal cells have been described namely light granular, dark agranular and basal cell (Shackleford and Wilborn, 1970 b).

The cell nucleus is characteristically centrally or apically situated in these ductal cells. The cytoplasm in the supranuclear region of the striated duct cell contains a few mitochondria, some rough endoplasmic reticulum and lysosomes. Microfilaments are abundant apically and golgi complexes may be numerous.

Although adrenergic nerves have been found around striated ducts in some species (Emmelin, Garrett and Ohlin, 1968 ; Garrett, 1974) they have not been found near these ducts in the rat (Norberg and Olson, 1965). In the adult mouse, axons have been observed intermingling with basal infoldings although direct endings on the striated duct cells have not been seen (Coughlin, 1975 a).

e) Excretory duct cells

The ultrastructure of these cells varies with the excretory ductal cell type present. In the rat submandibular

gland both in the main excretory duct and its branches light cells, dark cells and basal cells can be distinguished (Tamarin and Sreebny, 1965 ; Shackleford and Schneyer, 1971). Both light and dark cells have extensive infoldings in their basal membranes with tight junctions being found between cells. Basal cells have much less infolding in their basal membrane and contain hemidesmosomes.

Investigations of mouse submandibular gland excretory duct cells from an innervation viewpoint have been limited. In the cat submandibular gland cholinergic nerves are abundant around the excretory ducts (Garrett, 1966).

f) Myoepithelial Cells

These stellate cells surround the basal surface of acini, intercalated ducts and granular tubules but not striated or excretory ducts (Krause, 1865). In the rat submandibular gland the cells take up two basic shapes depending on whether they are located on end pieces or intercalated ducts (Tamarin, 1966). At end pieces they have extensive radiating cell processes whilst around intercalated ducts they are spindle-shaped and their processes rarely divide. The myoepithelial cell may indent the cell surface and have desmosomal contacts with it.

Ultrastructurally, myoepithelial cells resemble smooth muscle cells and react immunologically similarly (Archer and Kao, 1968 ; Archer, Beck and Melvin, 1971). Intracellularly the usual organelles are mostly juxta-nuclear but the most conspicuous feature is cytoplasmic filaments. These filaments are tightly packed bundles generally orientated in a longitudinal direction following the cell axis. They may be so tightly packed in some cell types as to appear homogeneous (Rhodin, 1962).

Myoepithelial cells are also present in embryonic rat submandibular gland in which myoepithelial cells arise by cytodifferentiation of epithelial stem cells in the distal buds and terminal tubules. Only the primitive cell can undergo mitotic division.

The principal innervation of rat myoepithelial cells appears to be sympathetic (Harrop and MacKay, 1968).

2.2.7 Ultrastructure of human minor salivary gland

As for murine salivary glands a knowledge of human labial cell ultrastructure and pattern of innervation has potential for cellular identification in mixed populations. Cell to cell contacts of the pattern and distribution of intracellular organelles may be important pointers to identifying a cell or its origin in a mixed culture *in vitro*. Similarly, a consideration given later of the pattern of innervation may be helpful in assessing the potential response of differential cell types to exogenously administered neurotransmitters.

a) Acinar cells

Acinar cells may be almost cuboidal in shape. The apical plasma membrane usually has no microvilli but secretory canaliculi are present (Tandler, Denning, Mandel and Kutscher, 1969 b). Desmosomes are present between acinar cells. Nuclei of acinar cells are centrally placed and the golgi cisternae prominent. These latter authors also alluded to functional morphological changes in these cells. During secretion, mucus drops previously noted to be membrane-bound in other species are released by an apocrine process (Martinez-Hernandez, Nakane and Pierce, 1972). These authors also suggest that in the mouse at least the plasma membrane has powerful membrane-lytic enzymes which dissolve the membrane immediately the blood flow is interrupted.

The neural innervation of human labial salivary glands was investigated by Tandler and Ross (1969). Intra-acinar nerve cell endings lacking schwann cells were described with myelinated and unmyelinated cells being found in the peri-acinar connective tissue. The intra-acinar nerve endings penetrated deep between cells and always lay close to myoepithelial cells.

b) Cells of the Intercalated duct

The transition from secretory end piece to intercalated duct cell is gradual (Tandler, Denning, Mandel and Kutscher, 1970) although the intercalated ducts themselves are of variable occurrence.

Intracellularly the nucleus is centrally placed and relatively few cytoplasmic organelles are found. Mucus containing duct cells are present and in these the golgi apparatus is prominent. Lysosome-like structures may be occasionally found in the apical cytoplasm of some cells.

c) Cells of the Striated and Intralobular ducts

Early electron microscopists considered there to be only one type of striated duct cell in human submandibular gland (Ferner and Gansler, 1961 ; Tandler, 1963). Latterly, ultrastructural studies on human labial gland have yielded two cell types (Tandler, Denning, Mandel and Kutscher, 1970). Both were basically similar with neither cell being granulated nor having a well-developed endoplasmic reticulum or golgi region. In both, the nucleus was centrally placed and numerous supranuclear mitochondria were present. On the basal membrane one cell had numerous infoldings and radially arranged mitochondria. The other lacked basal striations and had mitochondria randomly distributed in the basal cytoplasm.

d) Cells of the Excretory duct

These ducts are extremely short in labial salivary glands and show a variety of appearances depending on their proximity to the oral mucosa. A double layer of epithelium may be present and the distinction of cell type as used in other species into light, dark and basal cells may be applied (Shackleford and Wilborn, 1970 a, b).

Intracellularly, considerable ultrastructural specialisation can be seen and may be more complex than in striated duct cells. Of particular interest is the presence of hemidesmosomes on the basal aspect of basal cells.

e) Myoepithelial cells

Myoepithelial cells are present in human labial glands between the basal lamina and the acinar secretory cells (Tandler, Denning, Mandel and Kutscher, 1970). Intracellularly, numerous myofilaments have been observed being inserted into the basal plasma membrane. On the acinar cell surface, numerous desmosomes are present as well as apical cilia. These cilia on occasion extended deeply into an invagination of the secretory cell. In the non-filamentous region of the cell, endoplasmic reticulum, golgi bodies and lysosome-like bodies were also found. Clear cell myoepithelial precursors previously described by Tandler (1965) have been found sporadically between myoepithelial cell processes and secretory cells.

2.2.8 Conclusions

These anatomical considerations of human minor salivary glands and murine major salivary glands are of importance from two viewpoints :

(i) the cell type present and the distribution and type of intracellular organelles may have practical implications for identifying cells in a mixed culture *in vitro*. Identification of cells could be on an ultrastructural basis or on the basis of specific immunofluorescence reactions to intracellular components such as reaction to smooth muscle antibody or actin in myoepithelial cells.

(ii) in evaluating the response of cells to neurotransmitters *in vitro* consideration of the neural innervation of ductal and acinar cells is essential. In addition, the changing pattern of innervation during development (Yohro, 1970) must also be borne in mind. The response of a specific ductal cell type normally densely adrenergically innervated *in vivo* may be expected to differ from a normally sparsely innervated ductal cell type such as granular tubule cells.

CHAPTER 3

THE INFLUENCE OF NEUROTRANSMITTERS ON SALIVARY GLAND STRUCTURE AND DEVELOPMENT IN VIVO AND IN VITRO

3.1 INTRODUCTION

This section reviews the data to date concerning the action of neurotransmitters on murine major and human minor salivary glands. A considerable literature exists concerning the effects of neurotransmitters on the salivary glands of animals and man. The response of salivary gland tissue cultured *in vitro* has not been extensively studied and only a few papers, usually on diverse species, exist.

3.2 MURINE MAJOR SALIVARY GLANDS

The regulation of salivary gland size has intrigued numerous authors (reviewed by Schneyer, 1972). Here, an overall review is presented considering anatomical features before proceeding to molecular aspects of *in vivo* and *in vitro* studies.

3.2.1 Anatomical considerations

Several effects on salivary gland size have been observed to follow increased neural activity. Thus, the excessive neural stimulation following incisor amputation (Wells, 1963 ; Wells and Peronace, 1964) or feeding of a bulk diet (Wells and Peronace, 1967) increased parotid and submandibular gland size. Blockade of autonomic pathways (Brehner and Stanton, 1970) or surgical removal of the innervation (Wells, 1963) inhibits the increase in gland size demonstrating that acomplex neuronal pathway is anatomically related to the salivary glands and is mediated via neuro-effector sites in the glands (Emmelin and Holmberg, 1967).

Garrett (1972) reviewed the distribution of neuro-effector sites in the salivary glands of various species. This and later studies serve to illustrate the enormous variability between species, between salivary glands of the same species, between individual glands and the change of innervation with development time. In general, two types of neuro-effector sites are found in salivary glands. In one, interstitial axons have been observed to have a free surface in close proximity (circa 1000 nm) to parenchymal cells and in the other, axons are found beneath the parenchymal basement membrane with a smaller (200 nm) gap.

Histochemical studies of adrenergic sites have substantiated the anatomical studies in localising sympathetic endings in salivary tissue. It is known in rat submandibular salivary glands that dopamine-β-hydroxylase an enzyme involved in the synthesis of noradrenaline is mainly present around the acinar cells (Kato, Nagatsu and Nagatsu, 1974). Monoamine oxidase (MAO), involved in catecholamine metabolism, is present in the submandibular gland in the rat extraneuronally as well as in sympathetic nerves (Almgren, Anden, Jonason, Norberg and Olson, 1966). The exact site of MAO was not alluded to by these authors but the cytoplasm of acinar and ductal (unspecified) cells stained for MAO histochemically. This suggests that uptake into parenchymal cells of adrenergic neurotransmitter following neuronal release is one neuro-transmitter inactivation mechanism. Other studies also in rat submandibular gland localised extraneuronal catecholamine binding predominantly to striated duct cells under some conditions (Hamberger, Norberg and Olson, 1967). Catechol-O-methyl transferase (COMT) which is also involved in cate-cholamine degradation, is found in the parenchymal cells of rat submandibular salivary gland (Jonason, 1969 a).

The adrenergic receptors present in salivary glands show species differences (Emmelin and Holmberg, 1967) and in interpreting the results of <u>in vivo</u> studies the presence of

receptors on blood vessels or parenchymal cells must be borne in mind (Dirnhuber and Evans, 1954). In rat submandibular salivary glands in vivo beta-one type receptors (classification of Lands, Luduena and Buzzo, 1967) are present (Thulin, 1972).

Cholinergic transmission has also been studied in salivary glands predominantly in rat submandibular gland (Bogart, 1970, 1971). Acetylcholinesterase, involved in acetylcholine metabolism following neuronal release, was localised histochemically between adjacent acinar cells or between acinar cells and myoepithelial cells. This may not be a typical finding for the submandibular gland is unusual in that its secretory process is primarily regulated by the parasympathetic nervous system (Emmelin, Holmberg and Ohlin, 1965 ; Ohlin and Perec, 1965).

The predominant role of secretory regulation by the parasympathetic innervation of the rat sublingual salivary gland would be consistent with other in vivo studies. In these studies following the administration of noradrenaline, the catecholamine could not be detected by fluorescent techniques around acinar cells of the sublingual gland but was present around acinar cells of the parotid and submandibular salivary gland (Fujiwara, Tanaka, Honjo and Okegawa, 1965 ; Fujiwara, Tanaka, Hattori and Honjo, 1966). As yet there is little detailed information on the salivary cholinergic receptor.

3.2.2 Neural regulation of growth

Wells (1963) carried out much of the initial work concerning the role of neural innervation on salivary gland growth. Not only did repeated incisor amputation enhance submandibular salivary gland growth, but the effect did not depend on the integrity of the pituitary gland or the pituitary-adrenal axis. Histologically the increased size of the glands was attributed to an increase in size of acinar cells (Wells and Peronace, 1967). Later studies (Brenner and

Stanton, 1970) showed the gland weight increase to be due to an increase in cell number based upon total gland DNA content but this presupposes normal gland ploidy. Moreover, judged by beta blockade experiments using propranolol the receptor mediating this was a beta adrenergic receptor.

Using electrical stimulation *in vivo*, Matthews (1974) found that cells of the granular tubules responded to α sympathetic stimulation only in the presence of beta blockade. This finding was confirmed using sympathomimetic drugs by Hosoi, Aoyama and Ueha (1978) which required a prolonged time for recovery of secretory function (Azuma, Sato and Maruyama, 1980). Neural regulation of gland growth is not exclusively under sympathetic control since other authors have shown that preganglionic parasympathectomy produced submandibular gland atrophy (Snell, 1960) ; and also, *in vitro* salivary epithelium controls the axonal growth of parasympathetic nerves (Coughlin, 1975 b).

3.2.3 Drug induced salivary gland growth

3.2.3 (A) *In Vivo*

The finding that stimulation of nerves could enhance salivary gland growth is not unexpected for Selye, Veilleux and Cantin (1961) and Wells (1963) demonstrated salivary gland enlargement in rats following administration of the sympathominetic agent isoprenaline (isoproterenol). Histologically and biochemically (Barka, 1965) the increase in submandibular gland in size was attributed to an increase in size and number of acinar cells. In addition, Argonz (1962) reported the ineffectiveness of hypophysectomy in preventing this effect. Brown-Grant (1961) demonstrated that isoprenaline produces salivary gland growth in mice. In addition this author showed that, whereas in rats all three major salivary glands are equally enlarged (Selye, Veilleux and Cantin, 1961), in mice the effect is most striking in the parotid, less so in the submandibular and absent in the sublingual salivary glands.

Histologically, Brown-Grant reported a large increase in acinar size and that cells of the granular tubules appeared less prominent. Moreover, salivary gland iodine concentration relative to serum was unchanged in hypertrophic glands leading Brown-Grant (1961) to suggest a possible stimulatory effect on the tubule cells.

Drug isomers differ in their effects (Kirby, Swern and Baserga, 1969), such that in mice hypertrophy, hyperplasia and acinar cell damage are all part of the response to isoproterenol (Novi and Baserga, 1971 ; Simson, 1972). Studies since the 1960s have shown that there is a circadian rhythm to salivary gland uptake of tritiated thymidine and response to isoproterenol in mice (Burns, Scheving and Tsai, 1972). The stimulation of cell proliferation in mice by isoproterenol could also be detected in kidney (Malamud and Malt, 1971). Further studies have shown no change in salivary gland beta receptor number in rats following stimulation with isoproterenol (Burke and Barka, 1978). As would be expected from consideration of the postsynaptic siting of beta cell receptors, superior cervical ganglonectomy did not alter the response of rat salivary glands to isoproterenol (Barka, 1967). In addition, the growth response could be blocked by the beta-blocking agent propranolol but not the alpha blocker phentolamine (Sans, 1976). The enhanced growth response is a general beta receptor effect since other beta-agonists such as terbutaline have similar effects (Sodicoff, Sinesi and Pratt, 1980).

Electrical stimulation of the autonomic nerves to rat salivary glands showed differences in response between the parotid and submandibular salivary glands (Muir, Pollock and Turner, 1975 ; Templeton, Butcher, Turner, Muir and Durham, 1977). The parotid salivary glands responded to sympathetic stimulation by hypertrophy and hyperplasia whilst the response of the submandibular salivary gland was of hypertrophy only.

Ultrastructural studies in rats have given further evidence for the acinar cell effects of isoproterenol (Yamashina and Barka, 1972). By studying peroxidase-positive granules which in the developing rat submandibular gland are specific for acinar and proacinar cells (Dvorak, 1969 ; Strum and Karnovsky, 1970 ; Strum, 1971) depletion of these granules could be demonstrated following isoproterenol administration (Martin and Baserga, 1969). These granules are probably actively secreted by acinar cells since ultrastructural depletion of secretory residues also follows isoproterenol administration (Takahama and Barka, 1967).

Although these ultrastructural studies have demonstrated salivary secretion in response to isoproterenol, secretory function has been relatively understudied. In mice Brown-Grant (1961) observed no change in salivary gland amylase content following isoproterenol administration whereas Whitlock, Kaufman and Baserga (1968) and Ratti, Campora, Croce and Frati (1970) showed increased submandibular gland amylase levels but decreased esteroprotease levels following administration of isoproterenol to mice. In addition the activity of enzymes such as thymidine kinase paralleled DNA synthesis. In rat submandibular glands isoproterenol enhances protein synthesis (Ekfors and Barka, 1971), reduces the sialic acid content of the glands (Curbelo, Devalle, Houssay, Gamper and Tocci, 1968) whilst having no effect on arginase or acid phosphatase activity (Byrt and Glanvill, 1967).

Species differences may however produce differing results. In vivo, isoprenaline produced amylase secretion in large amounts in rats relative to the volume of saliva (Durham and Butcher, 1974). This study also illustrated the difficulty in interpreting the in vivo results of systemic drug administration. Pilocarpine, a cholinergic agonist produced salivary amylase secretion in lower amounts but this was via an indirect effect on the superior cervical ganglion. Schneyer (1965) previously observed modification of the action of pilocarpine on salivary secretion by adrenergic blocking agents.

Not only may drugs alter salivary gland responses *in vivo* but hormones may also play a role. Lacassagne (1940, a,b,c) first reported that mouse submandibular gland exhibited sexual dimorphism and that testosterone may stimulate the tubule portion of the gland of the female mouse such that it comes to resemble that of a male mouse. This was confirmed for specific tubule functions such as enzyme synthesis (Angeletti, Angeletti and Calissano, 1967). Hoshino and Lin (1970) demonstrated that by using isografts of submandibular gland there was a similar ductal growth in response to isoprenaline in denervated male and female grafts. Following testosterone therapy the female graft grew to resemble the male illustrating that neuronal connections are unnecessary for the hormonal response.

3.2.3 (B) *In Vitro*

Difficulty in interpretation of *in vivo* experiments has led some workers to consider the action of drugs on salivary gland growth *in vitro*. Malamud and Baserga (1967) were unsuccessful in attempting to maintain organ cultures of salivary glands from mice or rats of unspecified age. Kreider (1970) produced monolayer cultures from weanlingrat submandibular and parotid salivary gland. He demonstrated that isoproterenol at a dose of 50 $ngml^{-1}$ enhanced cell uptake of tritiated thymidine. He did not specifically identify an epithelial cell population although an acinar cell origin is implied for epithelioid cells present in the culture.

Single cell culture techniques have been able to sustain specialised cell types such as striated duct cells for periods of several hours (Kanamura, 1978). Cultures of dispersed rat parotid cells have been investigated and their response to clonidine and related imidazolines determined (Davis and Maury, 1978). Whilst not concerned with salivary gland growth, this study did demonstrate ion fluxes in response to alpha adrenergic stimulation. In addition, on the basis of blocking response to ephedrine, different groups of alpha

adrenergic receptors, some similar to those in brain, were described. The parotid derived cells were on morphological criteria acinar in origin (Mangos, McSherry and Barber, 1975 ; Kanagasuntheram and Randle, 1976).

Similar studies on other tissues in vitro such as frog skin have characterised the receptors involved in the glandular response to sympathetic stimulation as being alpha in nature (Benson and Hadley, 1969). It is of interest that although the site of action of sympathetic amines here appears to be glandular, in vitro attempts to localise noradrenaline uptake by salivary glands, have shown neuronal binding to be principally important (Sachs, de Champlain, Malmfors and Olson, 1970). This could represent a Type I transmitter uptake pattern following transmitter release (Iversen, Glowinski and Axelrod, 1966) although in vitro it is the salivary parenchymal cells which contain the major part of transmitter metabolising mechanisms (Jonason, 1969 a).

Other methods of tissue preparation in addition to the dispersed cell technique of Davis and Maury or slice type preparations of Sachs, de Champlain, Malmfors and Olson have been used to study salivary glands in vitro. An organ culture type of technique was used by Kaufman (1977) for whole tick salivary gland and was later modified by Denniss, Schneyer, Sucanthapree and Young (1978) to study the excretory ducts of rat and rabbit submandibular salivary glands. These authors were concerned principally with ion fluxes in response to sympathetic stimulation. Although not discussed in this paper, the apparent differences in response of ductal epithelium to sympathetic nerve activity and circulating catecholamines, namely altered ion flux and gland enlargement respectively, could be explained by parenchymal cell transmitter metabolism (Jonason, 1969 b), high affinity neuronal cell uptake (Sachs, de Champlain, Malmfors and Olson (1970), or a difference between short and long-term stimuli.

3.3 HUMAN SALIVARY GLANDS

Sialosis, or non-inflammatory, non-neoplastic recurrent bilateral swellings of the salivary glands (Thackray, 1972) occurs in association with a large number of conditions. The numerous causes have been classified by Rauch (1959) as hormonal, neurohumoral, dysenzymetic, malnutrition and drug induced. It is the latter group which are considered here as pharmacologically some may act as sympathetic or parasympathetic mimetic agents.

3.3.1 Anatomical considerations

Human major salivary glands have the same nomenclature as for the mouse. The submandibular and sublingual glands lie predominantly in the floor of the mouth whilst the parotid glands are preauricular in position and have superficial and deep lobes.

The majority of cases of major salivary gland enlargement involves the pre-auricular portion of the parotid gland (Mason and Chisholm, 1975). In hormonally induced sialosis, selective involvement of the submandibular salivary glands occurs (Thomson, McCrossan and Mason, 1974). Selective minor salivary gland enlargement does not appear to have been reported for hormonal or drug induced causes but does occur for some infections such as pulmonary mycoplasma infection (Wray, Scully, Rennie, Mason and Love, 1980).

3.3.2 Drug Induced Sialosis

A number of pharmacologically unrelated drugs cause sialosis (reviewed by Nash and Morrison, 1979 ; Banks, 1968). Catecholamine-induced sialosis by isoprenaline has been reported (Borsanyi and Blanchard, 1961). Histologically, the changes observed in the parotid salivary glands were similar to those induced experimentally using isoprenaline in rats.

3.4 CONCLUSIONS

In man and experimental animals sympathomimetic amines such as isoprenaline can produce salivary gland enlargement. Interpretation of the histological changes is discrepant with some authors claiming hyperplasia and hypertrophy as the processes involved in gland enlargement and others claiming hypertrophy as the only factor. Both species and glandular differences exist in response to isoprenaline and may explain these discrepancies.

The cell populations involved in either hypertrophy or hyperplasia have not been demonstrated by *in vitro* techniques. Nor has a direct action on gland cells by an agent producing these processes been reported. Selye, Veilleux and Cantin (1961) reported "intense mitotic proliferation of the serous, mucous and duct cells (in response to isoprenaline)" but this has never been followed-up by an *in vitro* study in which specific cell populations could be identified and the effect of neurotransmitters on growth evaluated. Later chapters of this thesis describe the techniques developed and results obtained in a study intended to achieve this aim.

CHAPTER 4

SPECIALISED PROPERTIES OF HUMAN AND MURINE SALIVARY GLANDS WITH PARTICULAR REFERENCE TO GROWTH FACTORS

4.1 INTRODUCTION

It has been customary to consider salivary glands as having principally an exocrine function. However in keeping with other glands which develop as outgrowths from the muscoa of the alimentary tract, such as pancreas, an endocrine function has also been attributed to the salivary glands.

Ito (1960) suggested the parotid salivary glands as perhaps being the most important salivary glands in this respect, claiming to isolate parotin. Recent work doubts the significance of parotin and studies have concentrated on the submandibular salivary gland. This chapter concentrates on growth factors isolated from human and murine submandibular salivary glands.

The relevance of including a review of these factors is two fold. Firstly the best characterised factors such as epidermal growth factor and nerve growth factor have a known neuronal stimulus to secretion. Events at nerve endings and following growth factor release may interplay in the control of cell differentiation. Secondly the epithelial-mesenchymal interactions leading to differentiation have a variable outcome depending on whether other factors such as mesodermal growth factor are present.

4.2 EPIDERMAL GROWTH FACTOR (EGF)

4.2.1 Identification of EGF

Cohen (1960) noted that when partially purified extracts of adult male Swiss-Webster mouse submandibular salivary glands were injected into newborn C.F.1 mice, several changes occurred. Daily subcutaneous injection of microgram

amounts of extract resulted in precocious eyelid opening (day 7 instead of day 12-14) and advanced incisor teeth eruption (day 6-7 instead of day 8-10). When female Swiss-Webster mice were used as the source of the extract the same dose by the same route did not affect eyelid opening or tooth eruption. The factor was present in a greater concentration in the male glands. It had the properties of a protein and as its principal effect was on epithelium it was termed Epidermal Growth Factor or EGF (Cohen, 1960). Species differences were also apparent, for when adult male rat submandibular salivary glands were used as the source of the extract. a negligible effect was observed on eyelid opening and incisor eruption in newborn C.F.1 mice. Extracts from a variety of other tissues had no effect on eyelid opening or tooth eruption following inoculation into newborn mice.

EGF was found to be a heat-stable, non-dialyzable antigenic protein of estimated molecular weight 15,000 daltons (Cohen and Elliott, 1963) which lacked both lysine and phenylalanine. Byyny, Ortho and Cohen (1972) later showed that the male submandibular salivary gland of Swiss-Webster mice contained 1000 ng EGF/mg wet tissue whereas adult female gland contained only 70 ng EGF/mg wet tissue. Roberts (1976 a) investigated EGF levels in the submandibular salivary glands of congenitally athymic (nude) mice. These mice have, in addition to thymic aplasia a deficit of hair formation and of ectodermal appendages in general. The normal development of the submandibular salivary gland from oral epithelium made this study of interest. There may be a relationship between the submandibular salivary glands and regulation of the immune system, since Cohen (1960) had also noted that the extract introduced subcutaneously also produced inhibition of hair growth and markedly stunted whole body growth of the animals. Roberts (1976 a) study found that the previously-observed sex differences in EGF levels were also present in athymic mice in similar ranges to those reported for other strains of normal adult mice.

Roberts (1976 b) studied the relationship between hyperplasia and EGF in the submandibular salivary glands of male BALB/mice using a single injection of isoprenaline. In addition, hypertrophy was induced by repeated isoprenaline injections or unilateral sialectomy. The EGF content of either or both glands was unchanged in hyperplasia but the EGF concentration was significantly reduced in hypertrophic glands.

4.2.2 Localisation, Chemical and Physical Properties of EGF

Cohen (1962), Jones and Ashwood-Smith (1970) and Cohen and Taylor (1974) all attempted to characterise further the initial crude isolate derived from male mouse submandibular salivary gland. Jones and Ashwood-Smith (1970) distinguished between epithelial growth factor and epidermal growth-factor (EGF) on the basis of molecular weight, electrophoretic mobility and chemical stability. Epithelial growth factor had a molecular weight of 22,000 and was heat-stable. This work draws attention to the effect of varying isolation procedures on the biological activity of the fraction isolated. Epithelial growth factor is an ill-understood poorly defined entity. Banks and Walter (1972) considered epithelial growth factor an artefact. On contrast to the earlier estimate of a molecular weight of 15,000 daltons (Cohen and Elliott, 1963) for EGF, Cohen and Taylor (1974) suggested a minimum molecular weight of 6,166 to 6,554 daltons. A single chain 53 amino acid polypeptide structure was deduced and the absence of the amino acid residues of alanine, phenylalanine and lysine confirmed. The factor was antigenic, heat-stable, non-dialyzable and also existed in a high molecular weight form (Taylor, Cohen and Mitchell, 1970). Phylogenetically this high molecular weight form is of interest since the binding protein is an arginine esterase of molecular weight 30,000 daltons.

Bradykinin (Schachter, 1969) and insulin (Chancre, Ellis and Bromer, 1968) have both been shown to arise from precursors by the proteolytic action of arginine esterases

suggesting a family of these enzymes may be involved in the activation of polypeptide hormones. Ekfors and Hopsu-Havu (1971) demonstrated six esteropeptidases in the adult male mouse submandibular salivary gland, one of which had the same ductal localisation as EGF.

Much work on the synthesis and localisation of EGF followed from the observation that the EGF content of the submandibular gland closely paralleled the development of its ductal system (Cohen, 1965 ; Roberts, 1974). Using immuno-fluorescent and autoradiographic techniques in vivo and in vitro, Turkington, Males and Cohen (1971) were able to demonstrate that EGF is both synthesised and stored in the ductal tubule cells of the mouse submandibular salivary gland. Van Noorden, Heitz and Pearse (1977) localised the site of EGF production in the mouse submandibular salivary gland to the granular tubules. The different levels of EGF in the male and female mouse are probably related to the sexual dimorphism exhibited by these granular tubules (Lacassagne, 1940 a,b,c).

The difference in EGF levels between male and female mice during postnatal development was investigated by Gresik and Barka (1978). The authors found that EGF was detectable in the cells of the granular convoluted tubules of male mice at twenty days postnatally and in female mice at thirty days. This contrasts slightly with the findings of Cohen and Taylor (1974) who found an exponential rise in EGF content from postnatal day 15 in male submandibular gland which is histologically mature at this time. A plateau level of 1000 ng/mg wet tissue (0.5% of gland wet weight) was reached on day 50.

Van Noorden, Heitz, Kasper and Pearse (1977) localised the site of EGF further in an ultrastructural study of the ductal cells in mouse submandibular salivary gland. Two populations of granules containing EGF were present in the granular convoluted tubule cells of the male mouse. In the female mouse when pregnant, the EGF levels equalled that of the male but gradually declined over the three weeks of

lactation. Whether this decrease is related to an increased activity of the 17β hydroxysteroid dehydrogenase in the female gland rapidly converting testosterone to androstenedione (Baldi and Charreau, 1972) which in turn produces cell division in the granular tubules or to the high (200 ng/ml) EGF content of mouse milk (Cohen and Taylor, 1974) resulting from systemic transfer of salivary gland EGF, is not known. Administration of androgens to female mice increases both the number of granular convoluted tubule cells (Grad and LeBlond, 1949 ; Baker, Chapp and Wardlight, 1964) and the EGF content of the gland (Byyny, Orth , Cohen and Doyle, 1974).

In the human submandibular salivary gland, Elder, Williams, Lacey and Gregory (1978) found h-EGF to be specifically localised in ductal cells. (Granular tubules are not present in human salivary glands). An additional finding in the human was that h-EGF showed a cross reaction with Brunners glands in the duodenum. Another polypeptide, urogastrone, is present in these glands and shares a number of biological properties with EGF including the ability to induce early eyelid opening in mice (Gregory, Bower and Willshire, 1978). Human urogastrone shares a sequence of 37 amino acids in common with h-EGF (Gregory, 1975). Mouse arginine esterase converts human epidermal growth factor/urogastrone to a small molecular weight form (Hirata and Orth, 1979).

4.2.3 Stimulus to Secretion of EGF

The factors controlling EGF secretion are best studied by *in vitro* techniques. Roberts (1977) using the sub-mandibular salivary glands of adult male BALB/C mice, was able to show an order of magnitude increase in EGF secretion in response to adrenergic agonists, and which was inhibited by alpha blockers such as phentolamine. Colchicine, which inhibits microtubule activity, reduced secretion thus suggesting it to be an active process involving microtubules.

Footnote : h-EGF refers to human EGF ; EGF is used to refer to mouse EGF being prefixed m-EGF where especial clarification is required.

Byyny, Orth, Cohen and Doyle (1974) in a detailed *in vivo* and *in vitro* study of mouse salivary glands were able to show that electrical stimulation of the superior cervical ganglion or administration of alpha agonists elevated the serum EGF levels from 1 ng/ml to 150 ng/ml in one hour and concurrently reduced the submandibular salivary gland levels of EGF. Since EGF is a polypeptide this stimulus to secretion is in keeping with other proteins secreted by the submandibular salivary gland which are principally secreted in response to adrenergic agonists (Byrt, 1966 ; Schneyer and Schneyer, 1967). The superior cervical ganglion is also involved in the maintenance of circadian periodicity exhibited by EGF. EGF concentration in mouse serum does not exhibit a circadian periodicity but the gland content of EGF does (Kreifer, Hauser, Liotta and Zelenetz, 1976). Salivary EGF levels were not measured.

Cholinergic agents such as carbachol (Byyny, Orth, Cohen and Doyle, 1974) do not alter serum EGF levels, although *in vivo* salivation is increased. Roberts (1978) found extracellular Ca^{2+} is necessary for EGF secretion *in vitro*. No studies of EGF secretion have followed the use of a sensitive radioimmunoassay for EGF (Byyny, Orth and Cohen, 1972) to show that the parotid salivary gland and pancreas both contain EGF albeit in very low concentrations (0.21 - 0.4 ng/mg wet tissue) (Cohen and Savage, 1974).

4.2.4 Biological role of EGF

EGF has been implicated in physiological events and pathological states in both the mouse and the human.

(A) Relationship between EGF and tissue function

The suggestion has been made that EGF may play a role in epithelial differentiation and development (Carpenter, 1978). Epithelially derived structures such as ductal elements in a number of tissues including breast are also

stimulated into cell division by EGF (Turkington, 1964, 1969).

The relationship between the development stage of a tissue and its response to EGF has not fully been elucidated. In humans the cord venous blood concentration of EGF is equivalent to the serum concentration (Ances, 1973) transplacental transfer remaining an open possibility.

Cohen (1965) has monitored the EGF content of the submandibular salivary gland and has shown maternal transfer of large amounts of EGF in milk. Bedrick and Ladda (1978) injected EGF in a concentration 40 μg per g animal into pregnant mice and found an increased frequency of cleft palate formation in offspring. Transplacental transfer of EGF appears to occur. An increased incidence of cleft palate formation was observed in the offspring but this only reached statistical significance when cortisone was simultaneously administered. With this inoculation regime cleft palate incidence reached 90% in offspring. These experiments are difficult to interpret but may provide further evidence for the role of EGF in the development of epithelially derived tissues for failure of palatal fusion may be the result of premature epithelial maturation.

Antisera to EGF may be expected to provide useful insight into the role of EGF. Mann and Fenton (1970) in a paper entitled 'Epithelial Growth Factor', but which refers to epidermal growth factor, found that the antisera did not prevent the action of EGF but in some cases the epidermis was thicker than with EGF alone.

Birnbaum, Sapp and Moore (1976) attempted to investigate the molecular basis for EGF action using epidermal mitosis as a model system. The authors demonstrated that <u>in vitro</u>, EGF stimulated epidermal mitotic activity and this was inhibited by elevators of cyclic AMP levels. This evidence is consistent with other <u>in vivo</u> evidence (Frati, D'armiento, Gulleta, Verna and Covel, 1977) which indicated that EGF decreased adenyl cyclase activity and cyclic AMP concentration in mouse

epidermis after several days. During in vitro studies on skin (Hoober and Cohen, 1967 a,b) EGF was found not to alter DNA synthesis but did stimulate RNA and protein synthesis. A number of molecular ribosomal mechanisms for EGF action are speculated with the induction of ornithine decarboxylase acting as a secondary event (Cohen and Taylor, 1974).

Molecular aspects of m-EGF binding to cells have been investigated (Das and Fox, 1978 ; Schlessinger, Schechter, Willingham and Pastan, 1978 : Schechter, Hernaez, Schlessinger and Cuatrecasas, 1979). Such studies have shown that a hormone-receptor complex is formed by m-EGF when binding to mouse fibroblasts (Schechter et al., 1979) and finally that the level of receptor occupancy is related to stimulation of DNA synthesis in the cell (Carpenter, 1978 ; Das and Fox, 1978). It is of interest in view of a possible common phylogenetic mechanism that insulin is bound and internalised in a similar way (Hollenberg and Cuatrecasas, 1973) as is thrombin (Baker, Simmer, Glenn and Cunningham, 1979). Certain amines inhibit the receptor clustering that occurs prior to internalisation (Maxfield, Willingham, Davies and Pastan, 1979).

Human EGF has also been studied (Carpenter and Cohen, 1978). Human EGF administered to mice mimicked mouse EGF in its effect on eyelid opening and tooth eruption (Cohen, 1962). Differences in composition were present between the two species since human EGF lacked theonine whilst mouse EGF lacked phenylalanine, alanine and lysine. As in the mouse, basal serum levels of h-EGF are present within a range of 2-4 ng ml with salivary (mixed) levels of 3-17 ng ml. No diurnal rhythm exists for serum h-EGF (Starkey and Orth, 1978).

The siting in the submandibular salivary gland and in the duodenum of two related polypeptides (EGF and urogastrone) with known effects on epithelial proliferation invites speculation. The agents both may act at the two sites on the gastro-intestinal tract which most frequently experiences injury to the mucosa ; that in the mouth occurs from the

trauma of mastication and that in the duodenum from gastric acid injury. It is not known whether EGF and urogastrone (which may be the same as human epidermal growth factors h-EGF) may act topically following release into saliva or duodenal secretions, or systemically since serum levels in the nanogram range are present for both substances.

(B) Relationship of EGF to the tumour state

Experiments involving chemical carcinogens and virally transformed cells have implicated EGF as enhancing tumour development. Reynolds, Boehm and Cohen (1965) and Rose, Stahn, Passovony and Herschman (1976) showed that concurrent administration of EGF with skin carcinogens increased the tumour frequency and reduced the latent period of tumour development.

Todaro, De Larco and Cohen (1976), Todaro, De Larco, Nissley and Rechler (1977) and De Larco and Todaro (1978) showed that in mouse cells transformed with a DNA virus the cells retained the ability to bind EGF whilst transformation by an RNA virus led to loss of the ability to bind EGF. Pruss, Herschman and Klement (1978) showed that the presence of an EGF receptor on the cell was not necessary for RNA viral transformation and Pratt and Pastan(1978) demonstrated that a surface glycogen may be necessary for EGF binding. The inter-relationship between cell transformation by viral or chemical means and EGF has still to be elucidated but it is known (Turkington, 1969) that EGF can stimulate proliferation of mammary carcinomas *in vitro*.

4.3 NERVE GROWTH FACTOR (NGF)

4.3.1 Initial observations.

4.3.2 Localisation, Chemical and Physical properties of NGF.

4.3.3 Stimulus to secretion of NGF.

4.3.4 Biological role of NGF

(a) Relationship between NGF and tissue function

(b) Relationship of NGF to the tumour state

4.3.1 Initial Observations

Bueker (1948) discovered a factor which was involved in the growth response of sympathetic nerve cells. Implanting mouse sarcoma cells (type 180) into the body wall of three day old chick embryos resulted in enlargement of sensory ganglia which innervated the implanted region. Later experiments showed that the growth enhancing effect was even greater in sympathetic ganglia and was not confined to the area innervating the tumour. A diffusible factor released by the tumour and which had a trophic response for sympathetic nervous tissue was postulated. This factor was later termed simply nerve growth factor (NGF) although at that time it was thought to be predominantly a growth factor for sympathetic neurones.

A much more potent source of NGF was discovered by Levi-Montalcini (1958) and Levi-Montalcini and Cohen (1960) to be mouse submandibular glands. Cohen (1960) showed that by administering an antisera specific to NGF into newborn animals, including the mouse, the sympathetic ganglia were destroyed or failed to develop. It was whilst studying NGF that Cohen discovered EGF.

4.3.2 Localisation, Chemical and Physical Properties of NGF

NGF is absent from the submandibular glands of newborn mice, i.e. at a time when the tubules have not yet undergone differentiation. This, coupled with the fact that in adult animals the male has a concentration of NGF ten times that of the female animal (Cohen, 1965) and androgen-dependance of the granular convoluted tubules (Lacassagne, 1940 a ; Junquiera, 1951) is further indirect evidence for the localisation of NGF in the convoluted tubules of these glands. Using an immunoperoxidase technique it has been shown that mouse NGF is exclusively localised in the granular tubule cells of the submandibular salivary gland (Schwab, Stockel and Thoenen, 1976). NGF is a protein which exists in a number of forms

(Varon, Nomura and Shooter, 1967), including a high molecular weight form of 140,000 daltons. This form of NGF, designated 7sNGF, was later shown by the same authors to be a complex of three subunits, alpha, beta and gamma. The biological activity of NGF resides in the beta subunit and has been called 7.5sNGF. The beta NGF subunit is a dimer whose primary structure has been elucidated (Angeletti and Bradshaw, 1971).

4.3.3 Stimulus to secretion of NGF

As with EGF, large sex differences exist in the levels of NGF isolatable from male and female adult mouse submandibular glands. The concentration in the male is ten times that of the female. Injection of testosterone results in a marked increase in NGF content in the female gland (Caramia, Angeletti and Levi-Montalcini, 1962 ; Levi-Montalcini and Angeletti, 1964). Surgical removal of the submandibular salivary gland in adult mice of both sexes causes a profound drop in plasma level of NGF ; this drop however is transient suggesting extra-salivary sites produce NGF in a similar way to EGF. Sialectomy also reduced tyrosine hydroxylase activity within sympathetic ganglia.

Current evidence suggests that NGF is synthesised and stored in the submandibular salivary gland (Burdman and Goldstein, 1965). Schwab, Stockel and Thoenen (1976) suggested that since NGF was immunochemically detected at the apical but not the basal part of the secretory tubule cells, then during transport from base to apex of the cell NGF may be transferred from a precursor molecule into immunologically reactive NGF. Binding proteins or additional short sequences may sterically hinder access of antibodies to NGF until just before secretion. These authors also found that the parasympathomimetic agonist carbachol resulted in massive NGF release into the salivary duct but could not detect NGF in whole saliva. The authors concluded that NGF must be rapidly broken down in saliva. Several other possibilities must exist to explain this phenomena including oral and ductal epithelial

binding of NGF, uptake by periductal lymphatics or binding to other proteins in saliva. More recent studies have shown NGF secretion into mouse saliva by alpha adrenergic stimulation (Wallace, Parthow and Wardell, 1977). Nearly all NGF in saliva is in the 7s form (Burton, Wilson and Shooter, 1978).

4.3.4 Biological effects of NGF

(a) Relationship between NGF and tissue function

Since antisera to NGF result in failure of maturation of sympathetic ganglia a trophic role in the maintenance of ganglia (predominantly sympathetic) has been postulated. Surgical removal of the submandibular salivary gland causes a profound drop in plasma and tissue concentrations of NGF. Other sites for NGF synthesis exist since the serum level of NGF following sialectomy gradually returns to normal. These sites are not known but are not the parotid and sublingual salivary glands. Submandibular salivary duct ligation in mice resulted in a decrease in gland NGF concentration (Weis and Buecker, 1966) as does castration. Interestingly, gonadal atrophy results from submandibular salivary gland ablation in male guinea pigs but not in rats (Junqueira and Fava-de-Moraes, 1965 ; Suzuki, 1972). The mechanism is unknown.

The molecular basis for NGF action is not known. Enhancing effects on glucose metabolism (Cohen, 1959) lipid metabolism (Luizzi, Angeletti and Levi-Montalcini, 1965) and protein and RNA synthesis (Cohen, 1959) have been established. This pleiotypic response of NGF may be the result of it having a primary structure similar to insulin (Bradshaw, Hogue-Angeletti and Frazier, 1974).

In nerve cells *in vitro* NGF rapidly increases intracellular cyclic AMP (Schubert, La Corbiere, Whitlock and Stallcup, 1978). This in turn mobilises calcium ions and causes an increase in cell to cell and cell to substrate adhesiveness, the latter being important for neurite extension

in vitro. The ability of NGF to adhere to surfaces used in *in vitro* studies was investigated by Pearce, Banthorpe, Cook and Vernon (1973) who considered its high affinity for such surfaces to have consequences for NGF activity as reported in the early literature.

(b) The Relationship of NGF to the tumour state

The initial observation by Bueker (1948) showed that NGF can be produced by some tumours such as sarcomas, and that NGF released from such tumours is active *in vivo*. Since then NGF synthesis by continuous cell lines such as 3T3 and SV3T3 has been established (Oger et al. 1974). In mice Furukawa, Nitishani and Hayushi,(1977) have shown that in the submandibular salivary glands of mice with neurological disorders, such as muscular dystrophy, there is a deficiency of NGF. The relationship between NGF deficiency, dysfunction of sympathetic ganglia and the innervation of skeletal muscle is unclear.

Human patients with neuroblastomas contain a factor in their ascitic fluid which behaves like NGF in its biological and chemical characteristics (Lyon, 1970).

4.4 THYMOCYTE TRANSFORMING FACTOR (TTF)

4.4.1 Initial Observations

A number of observations suggested that a factor, or factors,in the mouse submandibular salivary gland regulated immune responsiveness. Levi-Montalcini and Cohen (1960) found that by injecting crude extracts (lacking EGF and NGF) of mouse submandibular salivary glands into newborn mice, the phenomenon of runting occurred.

Takeda and Grollman (1968) found that injections of gland extracts produced thymic atrophy and lymph node atrophy when injected into newborn mice. The atrophy resulted from a reduction in the small lymphocyte population of lymphoid tissue. This effect was apparently independent of the adrenal cortex and gonads because the extracts still produced lymphoid atrophy in adrenalectomised or gonadectomised mice. In addition the same authors found that removal of the submandibular salivary gland delayed involution of the thymus.

Martinez-Hernandez, Nakane and Pierce (1973) however found that submandibular salivary gland removal in rats and mice resulted in a predominantly T-lymphocyte depletion of spleen and lymph nodes as well as thymic atrophy. Intraperitoneal injections of submandibular salivary gland homogenates prevented such atrophy.

4.4.2 Localisation, Chemical and Physical Properties of TTF

TTF is less well characterised than EGF or NGF. Naughton et al. (1969) and Naughton, Geczy, Hoffman and Hamilton (1972) determined it to be a homogeneous protein which had esteroprotease (enzyme esterase plus protease) activity. TTF has no EGF-like action. The site of synthesis of TTF is unknown, nor is it known if TTF is simply stored in the submandibular salivary gland. The relationship of TTF to thymosin and other thymic hormones has not been determined (Schlesinger and Goldstein, 1975).

4.4.3 Stimulus to Secretion of TTF

Factors affecting TTF secretion have not been investigated to date.

4.4.4 Biological Effects of TTF

A role for TTF in the immune response in mice has been postulated. Roberts, Freston and Reade (1976) however found

that extirpation of the submandibular salivary gland did not affect the delayed hypersensitivity response. This conflicts with the work of Kongshavn and Bliss (1970) who found delayed rejection of skin allografts in mice following injection of submandibular salivary gland extracts. Since EGF could mimic the action of extracts on the delayed hypersensitivity reaction, and extirpation of the submandibular salivary gland does not abolish plasma EGF concentrations (Byyny, Orth, Cohen and Doyle, 1974) then extra-glandular sources of EGF could explain the failure to change the immune response by submandibular gland extirpation.

Naughton et al. (1969) demonstrated that a crude submandibular salivary gland extract could transform *in vivo* and *in vitro* small lymphocytes directly into plasma cells without cell division.

Direct comparison between these various authors is difficult since it is not clear if each author was dealing with a comparable submandibular salivary extract. For example, Roberts, Freston and Reade (1976) showed that tissue extract prepared from the submandibular salivary gland of adult male BALB/c mice depressed chemically induced delayed type hypersensitivity in mice but EGF could mimic this action.

4.5 MESODERMAL GROWTH FACTORS (MGF, F-2)

4.5.1 Initial Observations.

4.5.2 Localisation, Chemical and Physical Properties of MGF/F-2.

4.5.3 Stimulus to secretion of MGF/F-2.

4.5.4 Biological role of MGF/F-2.

4.5.1 Initial Observations

Of all the growth factors isolated from the submandibular salivary glands of the mouse, probably least is known about this growth factor. A group which had previously worked with EGF and NGF (Attardi, Levi-Montalcini, Wenger and

Angeletti, 1965) described another growth factor different from these two. The factor was in the macromolecular fraction of the gland isolate and referred to as mesodermal growth factor. More than one factor may be present since Weimar and Haraguchi (1975) described another factor chemically different from any described and designated F-2.

4.5.2 Localisation, Chemical and Physical Proterties of MGF/F-2

Both MGF and F-2 can be isolated from whole gland homogenates using a Sephadex column technique and chromatography. No attempt was made in either of these studies (Attardi et al. 1965 ; Weimar and Haraguchi, 1975) to localise the activity of either fraction to any specific component of the gland although a non-epithelial derivation of both factors is implied.

4.5.3 Stimulus to Secretion of MGF/F-2

Details of the events governing the release of either factors have not been elucidated. Like the growth factors previously described, the biological effect of these factors is considered to be primarily but not exclusively on the tissue after which they are named. This activity may be evident during embryonic gland development as well as in the adult animal.

4.5.4 Biological Effects of MGF-F-2

MGF was found to have two main effects in mice. Firstly, if embryonic mesenchyme was separated from its epithelial component, such as bronchial epithelium, *in vitro* then the addition of MGF greatly stimulated the proliferation of any mesenchymal cells still attached to the epithelial component without any change occurring in the latter. Secondly, in tissue which had already undergone a degree of differentiation such as muscle or cartilage, then the addition of MGF *in vitro* appeared to prevent regressional change occurring in these tissues without cell division occurring.

Weimar and Haraguchi quantified the growth promoting ability of F-2 compared with EGF, NGF, MGF and TTF and found it to be as potent as MGF on some tissues. Interestingly from the phylogenetic viewpoint, these authors noted that all five growth factors possessed estero-peptidase activity (trypsin-like) but that this enzymatic activity did not appear to be related to the degree of growth stimulation at least in connective tissue cells.

4.6 CONCLUSIONS

These growth factors will no doubt be an area of increasing interest. At present no physiological or pathological function is directly attributable in humans or rodents to any particular growth factor. Only the "major" growth factors isolatable from the submandibular salivary gland have been discussed although others may exist (Angeletti, Salvi, Capani and Frati, 1965) ; Bast and Mills, 1968).

Much speculation, Rudland, Gospodarowicz and Seifert (1974), exists as to the function of these factors. They may represent a new class of polypeptide hormones whose primary action is in the control of differentiation and growth in different organs or tissues in the intact animal. The relative specificity of growth enhancing effects in different tissues of each factor reflected in their nomenclature may give support to this theory.

Although the present study is concerned with the effects of neurotransmitters on differentiation cognisance has been taken of the fact that other currently unmeasurable factors may also be active. This role of neurotransmitters as mediators of neuronal network is only part of the cell communication system involved in embryological development (Rutter, 1978).

CHAPTER 5

GROWTH OF SALIVARY TISSUE IN VITRO

Histologically, there are four main cell types in the adult salivary gland, excluding the vascular and neural elements : ductal, myoepithelial and acinar cells and fibroblasts. A problem is to decide how these may be individually cultured and identified.

5.1 IDENTIFICATION OF CELLS ARISING FROM CULTURE OF RAT AND MOUSE SALIVARY GLAND EXPLANTS

Culture of adult rodent salivary glands has been reported by numerous authors : thus, Trowell, (1959), Tapp (1967), and Lucas, Peakman and Smith (1970), using adult rat salivary glands and Vecchione (1967) using adult mouse salivary gland observed degeneration of acinar cells and hyperplasia of ductal elements after several days *in vitro*. Lucas (1969), using immature rats, noted that salivary glands from one week old animals differed from adult glands in their ability to survive *in vitro* ; parotid and submandibular salivary glands survived badly except while still largely undifferentiated (age 1-2 weeks).

In the rat submandibular salivary glands, the full range of differentiated cells present in the adult gland does not appear until about the tenth week after birth (Jacoby and Leeson, 1959). Two major elements, acini and granular tubules, are absent at birth. Mouse salivary glands have a predominantly prenatal development (Yohro, 1970) and may, therefore, be better for the *in vitro* study of salivary gland differentiation. The manner in which cell growth occurs from an explant is clearly complex, involving consideration of differentiation, de-differentiation, maturation and even selective death of specific cells from explanted tissue.

Cell culture of adult mouse and adult rat salivary gland has been reported (Kreider, 1970 ; Gallagher, Marsden and

Robards, 1971 ; Mercante, 1973 ; Brown, 1973 ; Kanamura and Barka, 1975 ; Wigley and Franks, 1976). None of these studies used newborn animals. Most authors believed the salivary gland cells in culture were acinar in type, and epithelial in origin, although evidence from one author (Brown, 1973) suggested a connective tissue origin based on the tumours which arose following treatment with chemical carcinogens. On the basis of proteolytic activity, which in vivo is specifically confined to granular tubule cells Shear (1972), Mercante (1973) and Wigley and Franks (1976) concluded that the cells which proliferated in culture were granular tubular in origin.

Only Wigley and Franks (1976) in a detailed paper have attempted to identify conclusively the individual cell populations present. Histochemical techniques using a duct specific enzyme, 11β hydroxysteroid dehydrogenase, were unsuccessful in this study. Ultrastructural characteristics of epithelial cells such as the presence of desmosomes were identified.

Identification of components of the gland derived from epithelium, namely ductal myoepithelial or acinar cells, in a cell culture population is a major problem. The increasing literature reference to epithelioid or epithelial-like cells is indicative of this, confirming an increasing view that cell morphology is an unreliable criterion for cell identification.

Few ultrastructural features of cells in culture are specific (Franks, 1972) and this applies to salivary gland cells. For epithelially-derived cells, cell-to-cell contacts can be of value in cell identification. A method of embedding explants with any associated cellular outgrowth in situ for transmission electron microscopy is required to provide information concerning cell-to-cell and cell-to-surface contacts.

An aspect of the present work therefore has been to seek ultrastructural features of cultured cells which would provide a simple aid to cell identification.

Of the three types of specialised cell junctions (Farquhar and Palade, 1963 ; Tucker, 1968) only the desmosome was investigated here. This was because, firstly, it is the best known junction of stratified squamous epithelium (from which ductal tissue originates) and secondly, because tight junctions have been shown to vary in some tissues with both the type of dehydration agent used (Johnston and Koots, 1967) and the procedure for staining the tissue (Brightman and Reese, 1969). The criteria for identification of desmosomes are well established (Odland, 1958 ; Stern, 1965).

Desmosomes with associated tonofilaments if found in a proportion of cells in the explant cellular outgrowth would indicate epithelial cell origin. A practical consideration is that in other species such as rabbit, desmosomes in non-keratinised oral epithelium (Chen, 1970) occupy less than 20% of the plasma membrane, so there may be a chance of missing attachments unless serial sections are examined.

Histochemical studies allow cell identification on the basis of function but the possibility of differing epithelial and fibroblast membrane permeability to components of the histochemical reaction remains. Nevertheless, a considerable advantage of the method is that it allows localisation of cells in relation to the overall cellular outgrowth as well as providing a means of quantifying the number of both positively reacting and negatively-reacting cells present.

Mercante (1973) investigated lactate dehydrogenase (EC.1.1.1.27) pyruvate kinase (EC.2.7.1.40) amylase (EC.3.2.1) and phosphofructokinase (EC.2.7.1.56) activity of mouse submandibular gland cells and mouse fibroblasts in vitro. With the exception of amylase these enzymes are not cell-specific though tissue specific isoenzymes are known. Even with amylase, fibroblasts had one twentieth of the activity of submandibular gland cells. Collectively, however, there may be value in determining whether patterns of enzyme activity differ between fibroblast and epithelial cell populations. Other enzymes such as arylsulphatase which have been thought

to be present in ductal cells in other tissues (Kostulak, 1977) were included in the present study.

Ductal epithelium in salivary glands converts cortisol to cortisone by means of 11β hydroxysteroid dehydrogenase (11βHSD) (Ferguson, 1967). The significance of this enzyme is twofold. Firstly, its continued presence in vitro should correlate with conservation of ductal epithelial cell function. Secondly, that the cell population is not fibroblasts.

An aim in the present study was therefore to determine the distribution of 11βHSD in cellular outgrowths from primary explant cultures of murine submandibular salivary gland. The question of whether it could serve as a quantitative measure of the number of cells having a ductal epithelial origin, was investigated. Also, the activity of 11βHSD may also permit quantification of ductal cells in a non-invasive manner, so conversion of cortisol to cortisone was studied in the growth medium.

Other features of ductal epithelial cells may be of value in cell identification. In humans, an antibody to salivary duct epithelium has been described in patients with Sjogren's Syndrome (Bertram and Halberg, 1964) and in 25% of patients with rheumatoid arthritis alone (MacSween et al. 1967). This reaction if non-species specific would be a useful adjunct to murine ductal epithelial cell identification. The principle of such reactions have long been recognised but only recently (Diaz and Marcelo, 1978) applied to cell culture although the potential seems considerable. It was felt important to study the application of enzyme histochemistry and immunofluorescence for identifying cell populations in culture because of previously held views that differentiated epithelium lost its specialisation rapidly in vitro (Sato, Zaroff and Mills, 1960 ; Sandstrom, 1965).

5.2 MATERIALS AND METHODS FOR CELL CULTURE

5.2.1 Animal Stock

Since considerable data is already available concerning mouse salivary gland growth and development, mice were used throughout. Initially mice of the C3H strain were used as claims had been made (Henson and Strano, 1972) that in their submandibular salivary glands serous, mucous and seromucous components were present and anatomically separable into individual lobules. This would have been advantageous in that it presented the possibility of studying each acinar cell type individually. This work confirmed the brief report of Wescott and Shannon (1974), that the original claim could not be substantiated. Both myself and these other authors find all three elements present within individual lobules of the submandibular gland. Accordingly, an inbred strain had no advantages so the breeding difficulties were avoided by using an outbred strain of mouse, CFLP, in all but these initial experiments.

Newborn male animals were predominantly used as donors of gland tissue, being less than 24 hours old at the start of the experiment. Breeding pairs of animals which produced these litters were housed two per cage. They received standard laboratory chow and tap water ad libitum. Each animal was inspected and weighed regularly and litter size was noted as this gave a simple guide to general health and well-being.

5.2.2 Salivary Gland Dissection Techniques

Newborn animals were removed from the parent's case and male animals segregated by inspection. Animals were killed by anaesthetising them in a small jar with ether. A fine wire mesh covered the cotton wool in the bottom of the jar to prevent the animals contacting the ether pad.

The animals once dead were pinned through the limbs to a cork dissecting board covered with Benchkote (Whatman, England) which allowed swabbing of the neck region with absolute alcohol to minimise bacterial contamination. The neck region was opened by a midline incision and skin laterally reflected to expose the salivary glands. Under a Leitz stereo dissecting microscope the three major pairs of salivary glands were identified and removed using fine forceps and curved scissors. The glands were placed at room temperature in McCoy's medium 199 (Gibco Biocult, Scotland) containing penicillin (100U ml^{-1}), Streptomycin (100 $\mu g ml^{-1}$) and 10 mM HEPES buffer. As in the adult animal the submandibular salivary glands are large paired structures lying on either side of the midline with the smaller sublingual salivary glands on their anterolateral aspect. The parotid salivary glands lie lateral to the submandibular glands and unlike the adult animal, rarely extend medially.

Although acinar development is minimal in newborn animals acinar types are recognisable by their staining reactions with haematoxylin. The sublingual glands are mucous in type, the parotid salivary glands are predominantly serous and the submandibular salivary glands predominantly serous. In the submandibular salivary glands there is a tendency to sero-mucous change in the adult animal.

5.2.3 Culture Media

The media screened for their ability to support salivary gland growth *in vitro* were the following commercially available media :-

A. 199 (041-1153)
B. MEM (041-1090)
C. Williams (041-2541)
D. Trowell's (041-1490)
E. Waymouth's (041-1220)

All media were supplied by Gibco-Biocult, Paisley, Scotland. The constituents of these media are listed in Appendix A. Media were supplied in 500 ml sterile volumes and stored at +4°C. Checks for fungal and bacterial contamination were carried out periodically.

5.2.4 Sera used as supplements for media

Early workers had qualitatively determined the ability of culture media to support growth and it was a common observation that growth is often enhanced if a small amount of natural fluid such as serum is added to the synthetic media.

The following sera were used in this study :-

(A) Newborn Calf serum

This was supplied by Gibco-Biocult in 100 ml volumes. This volume was aseptically aliquoted in 10 ml amounts into sterile universal containers (Nunclon, Nunc, Denmark) and stored at -20°C. When required containers were thawed to room temperature and used immediately. Studies using a HeLa cell line tested the growth-promoting ability of individual batches. Media from several batches, all tested for growth-promoting activity were used simultaneously to reduce inter-batch variation.

(B) Foetal Calf Serum

This was supplied by Gibco-Biocult in 100 ml volumes and by Sera-Lab, Crawley Down, Sussex in 20 ml volumes. Volumes of 10 ml were aliquoted in sterile universal containers and maintained at -20°C until required. Batches were previously virus and mycoplasma screened.

(C) Horse Serum

This was supplied by Gibco-Biocult in 100 ml volumes and stored as for foetal calf serum. Batches were mycoplasma screened.

(D) Human Serum

A volume of 50 ml of blood was aseptically obtained by venepuncture in the antecubital fossa. Samples were all from Australia antigen negative adult males. Following centrifugation at 500 g for 5 minutes, samples were aliquoted in 10 ml volumes and stored at -20°C. Checks for fungal and bacterial contamination were carried out routinely.

(E) Mouse Serum

Mouse serum was obtained by cardiac puncture of 6 week old male CFLP mice. Large numbers of mice were used to obtain serum volumes of 40 ml. This was aliquoted as for the previous sera and routinely tested for fungal and bacterial contamination.

5.2.5 Antibiotics, Enzymes and Salt Solutions

Antibiotics

Use of antibiotics is necessary when culturing salivary explants which have to be excised through skin or mucous membranes which are inevitably contaminated with micro-organisms.

Penicillin was used at a final concentration of 100U ml^{-1} and Streptomycin at 100 $\mu g ml^{-1}$ (043-5070). Both were supplied by Gibco-Biocult in 100 ml volumes. Aliquots of 1 ml were stored at -20°C until required.

Trypsin

Trypsin in a concentration of 0.25% (043-5050) was supplied by Gibco-Biocult in 100 ml volumes in unbuffered normal saline. The solution was aliquoted in 5 ml amounts, stored at -20°C and thawed to room temperature immediately prior to use. Cultures were trypsinised by decanting the growth medium, rinsing x2 in Dulbecco's phosphate buffer saline (PBS) and adding a trypsin solution in PBS. This was formulated by diluting the stock solution (0.25% w/v) into PBS to give a

working solution of 0.025% w/v. This was applied to cultures for up to 2 minutes. The detached cells were harvested by aspiration and centrifuged at 500 g for 3 minutes. The cell pellet was suspended in PBS and again centrifuged. The resulting washed cells were suspended in culture medium containing serum supplement to inhibit any remaining trypsin.

Salt Solutions (Dulbecco's phosphate buffered saline)

As supplied by Gibco-Biocult (code 041-4040) its composition is that of Dulbecco and Vogt (1954). The solution is composed of NaCl (6800 mg l^{-1}) KCL (400 mg l^{-1}) $MgSO_4 7H_2O$ (200 mg l^{-1}), $NaH_2PO_4 2H_2O$ (158 mg l^{-1}) $CaCl\ 2H_2O$ (264 mg l^{-1}), $NaHCO_3$ (2200 mg l^{-1}) Glucose (1000 mg l^{-1}) and Phenol red (10 mg l^{-1}). The pH of the solution was checked routinely prior to use and was 7.25 ± 0.1. Storage was at +4°C.

5.2.6 Preparation of Tissue for Cultures

In vitro culture of murine submandibular gland explants (1 mm^3) can result in tissue degeneration in a relatively short time (Trowell, 1961 ; Easty, 1970) but there has been little attempt to investigate systematically the relationship between explant size and continuing viability.

Initial explant size is therefore an important factor in explant viability. Investigation of this problem is reported in Chapter 6 using the following methods.

Newborn mouse submandibular glands were excised and temporarily placed in 199 transport medium containing Penicillin (100 μml^{-1}), Streptomycin (100 μgml^{-1}) and 10 mM HEPES buffer. Glands were finely chopped in this medium with curved scissors. A series of cuboidal explants were prepared whose diameters were measured with a gridded eyepiece and varied through the range 0.2 mm - 1.00 mm. It was found important for good attachment that the transport medium be tilted away from the submerged explants prior to their

removal from the first dish in order to minimise the amount of fluid carried over when placing them on the substratum for culture.

Two millilitres of growth medium (199 plus 20% newborn calf serum) was added to each 30 mm plastic petri dish (Nunclon, Nunc, Denmark) and the culture maintained in a CO_2 incubator (Grant Instruments, Cambridge) with a humidified atmosphere of 5% CO_2, 95% air at 37°C. Continuous cell lines of epithelial cells (HeLa) and mouse fibroblast cells (NCTC 929), both supplied by Gibco Biocult, were also grown under identical culture conditions. A human fibroblast-like cell line was obtained from the lamina propria of a gingival biopsy. The biopsy was grossly excised from its epithelial component and serially passaged five times under these standard conditions until a cell line with a fibroblast-like morphology (designated lamina propria culture) was achieved.

Human labial salivary glands were obtained by surgical removal of several lobules from the everted lower lip under local anaesthesia. The excised glands were placed in the transport medium and the wound closed with two interrupted sutures which were removed one week later when healing was satisfactory. Fifty four such biopsies were taken on approximately equal numbers of male and female patients with a mean age of 51.6 years.

5.2.7 Substrata

Six substrata were investigated :-

a. Detergent (Alconox Inc., New York) cleaned glass coverslips.

b. Carbon-coated glass coverslips.

c. Poly-l-lysine coated glass coverslips.

d. Polycarbonate (Thermanox, Lux Scientific Corp., U.S.A.).

e. Melinex (I.C.I., Welwyn Garden City, England).

The glass slides were coated with carbon after the method of Kay (1967) at a thickness of 0.1 μ. Poly-l-lysine (Sigma Chemical Company) was added to the slide with a pipette from a solution containing 75 mg per 100 ml water (modified from Mazia, Schatten and Sale, 1975) and the film allowed to air dry.

These slides except those coated with carbon were then briefly rinsed in distilled water prior to use. The substrata were sterilised either by dry heat (a and b) or ethylene oxide (c, d and e).

5.2.8 Culture Vessels and Incubation Procedures

Culture vessels employed were :-

(A) Petri dishes (Nunclon, Nunc, Denmark) of 30 mm diameter were air-vented and previously sterilised by irradiation.

(B) Tissue culture flasks (Nunclon, Nunc, Denmark) providing 25 cm^2 growth area were fitted with screw caps and rendered sterile by irradiation.

Preparation of tissue for culture was in a laminar flow cabinet.

Cultures were incubated in a constant humidity incubator at 37°C with a gas phase of 5% CO_2 95% air maintained by a carbon dioxide monitor (Gow-Mac Shannon, Ireland). A dual cylinder supply system was used with an automatic switchover.

5.2.9 Histological Techniques

It was first necessary to determine whether cell morphology at the light microscopic level could be used to establish cell origin in the cellular outgrowth. Submandibular salivary gland cultures in the growing and fixed and stained state were photographed using a Leitz Microscope with Leica camera attachment and Kodak 2483 colour positive film. Coverglass cultures were rinsed in phosphate buffered saline, fixed in 10% formal saline for one hour and stained :

haematoxylin and eosin were used routinely in the staining of all cultures. The findings are discussed in Chapter 6.

5.2.10 Cryostat Techniques

Frozen section material was required for both histochemical studies (5.2.12) and immunological studies (5.2.14). Newborn mouse submandibular salivary glands were used routinely. Dissection of the glands was as described and followed by freezing on solid carbon dioxide. Frozen tissue was mounted on a perforated chuck by the use of Tissue-Tek O.C.T. compound (Lab-Tek Products, U.S.A.). Sections were placed overnight in the cryostat (Bright Instrument Company, England). The following day 8 m thick sections were cut onto pre-cleaned glass slides (76 mm x 22 mm, Chance, England) and stored at -20°C until required.

5.2.11 Electron Microscopy

Salivary gland cultures were prepared for transmission electron microscopy after the method of Nopanitaya, Charlton, Turchin and Grisham (1977). Prior to fixation, cultures were washed twice in warm saline (37°C) for one minute each. Fixation was at room temperature for two hours in 2.5% glutaraldehyde in 0.2 M cacodylate/HCl buffer, pH 7.4 made freshly each time. They were then washed in 0.2 M cacodylate/HCl/8% (w/v) sucrose pH 7.4 for sixty minutes and treated at room temperature for thirty minutes in a 2% solution of osmium tetroxide in cacodylate/HCl/8% (w/v) sucrose buffer pH 7.4. These fixed cultures were briefly rinsed in distilled water and dehydrated in increasing concentrations of ethanol (50%, 75%, 95% and two changes at 100%). Following the second wash in absolute alcohol all cultures, with the exception of Thermanox-based cultures, were infiltrated in propylene oxide then 50:50 prolylene oxide and Durcupan Araldite mixture (containing equal volumes of resin and hardener) at room temperature. The propylene oxide step was omitted for

Thermanox-based cultures which passed directly from alcohol to resin before being placed in the Araldite mixture.

Glass coverslips were removed from cultures by the method of Moore (1975) using hydrofluoric acid dissolution of the coverglass from the epoxy-embedded cultures. The exposed surface of the culture was then embedded in Araldite and vertical sections prepared. Ultra-thin sections were then stained with uranyl acetate and lead citrate (Reynolds, 1963) and viewed on a Philips 301 transmission electron microscope.

Cell-to-cell contacts in the monolayer cellular outgrowth were then examined for specialised regions known to be characteristic of epithelial cells, namely desmosomes with associated tonofilaments (Farquhar and Palade, 1963).

5.2.12 Histochemistry

Localisation of 11β HSD (E.C.1.1.1.51) activity was used after the method of Baillie, Ferguson, Calman and Hart (1965). Cultures of newborn mouse submandibular gland were incubated for up to two hours in a phosphate buffered medium pH 7.4 containing NAD, nitro blue tetrazolium and 1 mM cortisol as substrate. Eight frozen sections of these immature glands were examined to confirm the ductal specificity of this enzyme.

Succinate (EC.1.2.4.2), isocitrate, (EC.1.1.1.42), α Ketoglutarate (EC.1.2.4.2) and glucose-6-phosphate dehydrogenase (E.C.1.1.1.49), arylsulphate (a and b, EC.3.1.6.1) were demonstrated using standard techniques (Ferguson, 1966 ; Pearse, 1972). Control sections were incubated concurrently in a medium without substrate and upon completion of the incubation the sections were washed in water and mounted using a glycerol:buffer medium (1:3 by volume). Sections were viewed immediately after mounting.

Cultures from the same gland were also stained using the periodic acid Schiff (P.A.S.) technique after the method of Hotchkiss (1948).

5.2.13 Autoradiography

These studies utilised tritiated thymidine (Radiochemical Centre, Amersham, England) at 1 μCiml^{-1} of specific activity 50 Cimmol^{-1} pulsed for six hour periods up to 24 hours on 4 day old cultures of newborn mouse submandibular gland. Standard criteria for cell labelling were adopted (De Robertis, Nowinski and Saez, 1948).

For other studies to attempt to identify epithelial or fibroblast cell populations specifically, tritiated tryptophan, tritiated proline and tritiated isoprenaline (Radiochemical Centre, Amersham, England) were used in a similar regime. Tritiated proline and tritiated tryptophan had a specific activity of 1 μCiml^{-1}. Pulse times from 4-48 h were used.

Proline was chosen since it is a precursor of collagen and would be expected to be taken up by fibroblasts. Tryptophan is known to be incorporated *in vivo* into the salivary granular tubule cells in rats (Materazzi, 1967) and isoprenaline would be expected to bind to surface receptors on which it was acting though it was recognised that this might not survive fixation.

The technique is illustrated in Figure 14 and consists of several steps :

1. On completion of the appropriate pulse time the coverglass is removed from the tissue culture medium (a, Fig. 14) containing the isotope and washed in phosphate buffered saline for a total of 15 min., with changes every 5 min.

2. Cultures are then fixed in 10% formaldehyde in phosphate buffered saline for 12 h.

3. The culture is washed in distilled water for a period of 30 min., with at least four changes. Steps 1 and 3 are assisted by gentle agitation.

4. The coverglass cultures are placed on clean filter paper with the cultured surface uppermost and any surplus distilled water allowed to dry but keeping the explant culture and cell surfaces moist with distilled water if required.

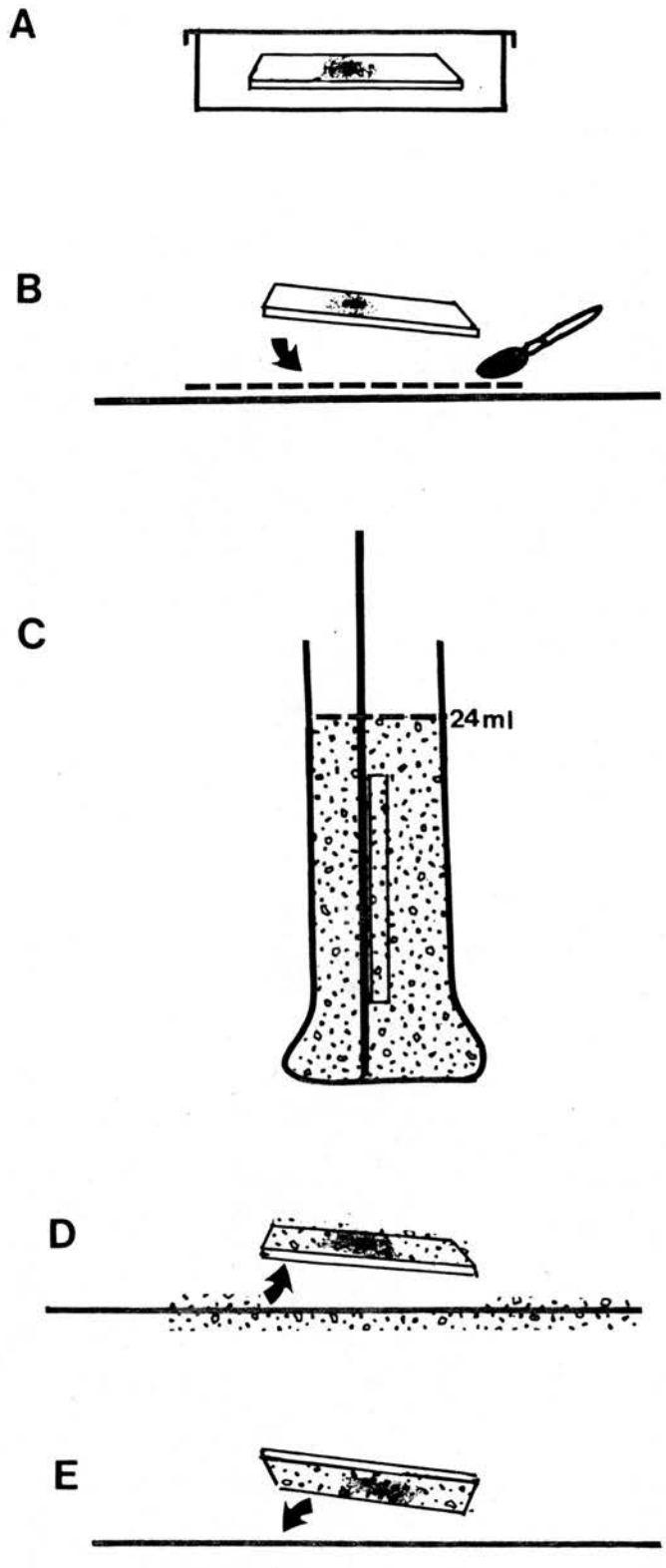


Figure 14

Outline of the steps involved in preparing cultures grown on coverglasses for autoradiography. Steps A - E are described in the text.

5. A corresponding number of clean dry microscope slides are prepared. This is also a convenient time to record any information or means of identification onto the slide by using a writing diamond.

6. Clear nail varnish is painted on to the slide in an outlined area corresponding to the size of the coverglass (b, Fig. 14). This area is best sited towards one end of the slide as it assists in emulsion coating. The coverglass culture is then placed on to the disc with the culture uppermost. The varnish acts as cement between the coverslip and disc and is allowed to dry (approximately 15-20 min.). Adherence of the coverglass to the microscope slide is adequately strong. It is important that the varnish is allowed to spread to the extreme edge of the coverglass, forming a complete seal. This prevents contamination of both sides of the glass by nuclear emulsion when dipping. It is very important that the culture surfaces are kept moist with distilled water to ensure preservation of photographic detail.

7. Ilford K5 nuclear emulsion (Ilford Limited, Ilford, Essex) is prepared in the dark and the dipping technique (c, Fig. 14) applied as a thin film as described by Rogers (1973).

8. After drying, the slide cultures are placed in a light-proof box containing silica gel for the duration of exposure at 4°C. For salivary gland culture the time required was 7 to 14 days.

9. On completion of exposure the slides are developed using D19 developer and fixed in Kodafix (Kodak, Hemel Hempstead).

10. After washing well in running water for a minimum of 30 min., the slide/cultures are stained by haematoxylin and eosin.

11. The stained preparations are dehydrated through alcohols and cleared in xylene. This was a convenient holding stage when processing a series of cultures.

12. Each slide was taken individually and immersed briefly in acetone to dissolve the varnish bond. By applying a sharp razor blade round the periphery of the coverglass, detachment can be hastened. Excess varnish should be removed from the coverglass with a paper tissue.

13. Following acetone cleaning the coverglass is again dehydrated through absolute alcohol and cleared in xylene.

14. The coverglass with the emulsion-coated culture side down is remounted on to a clean, dry slide using Kodak Harleco Synthetic Mounting Resin (Kodak, Hemel Hempstead).

15. It is advisable to leave the finished preparations overnight before viewing microscopically. This allows for proper penetration of the emulsion by the mounting medium.

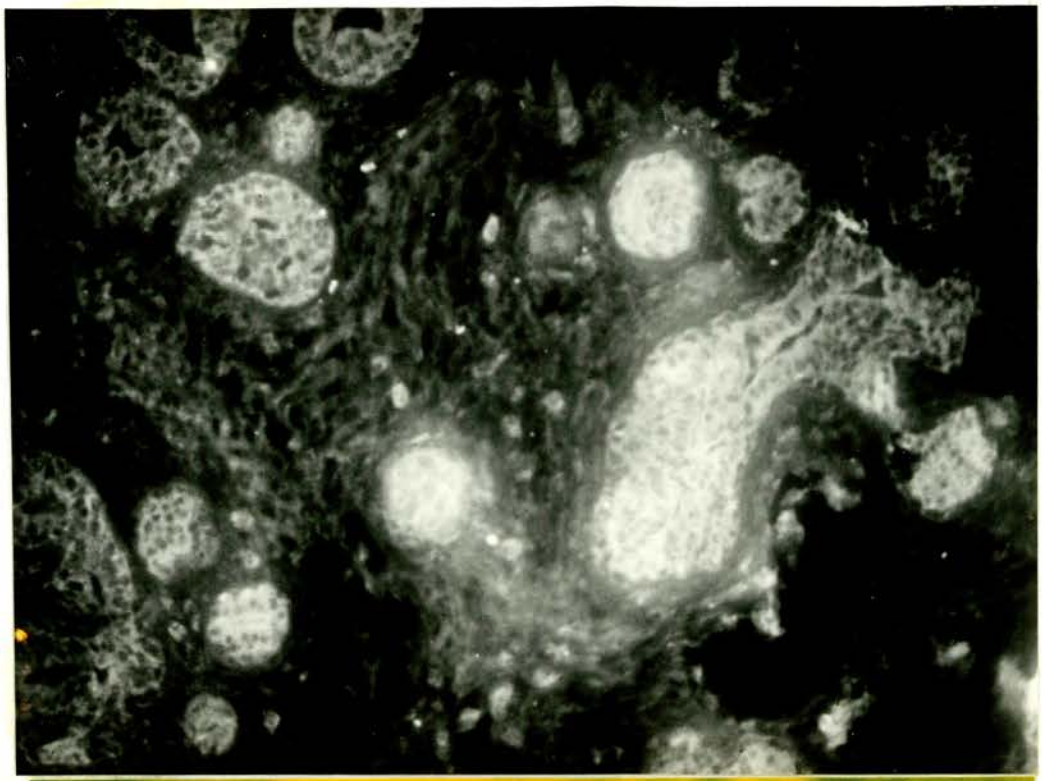
5.2.14 Immunofluorescence

Sera were obtained from patients with Sjogren's syndrome who were proven on two separate occasions to be strongly positive for circulating salivary duct antibody. Figure 15 shows the normal reaction obtained by an indirect immuno-fluorescent technique using frozen sections of human sub-mandibular gland. This antibody reacts with salivary ductal epithelial cells in a manner which is non-species specific.

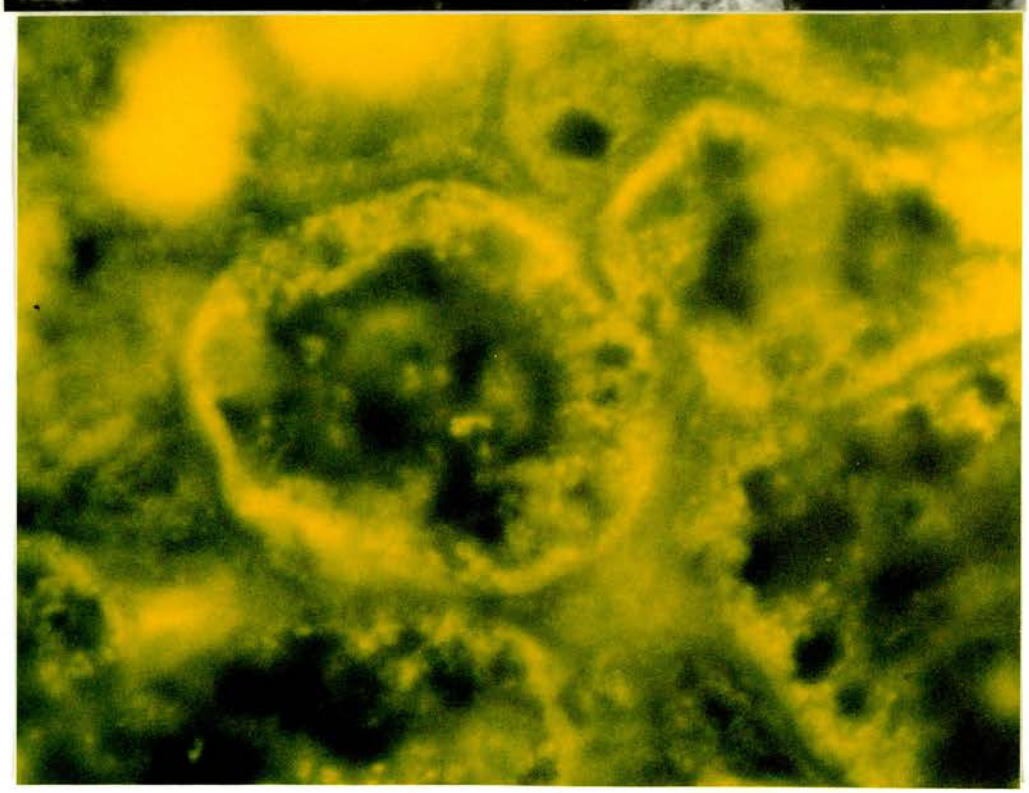
Seven day cultures of newborn mouse submandibular gland grown on coverglasses were incubated with undiluted patient and control sera at 20°C for 30 minutes, rinsed in two changes of 0.5 M barbitone buffer mounted in a glycerol medium and immediately photographed on a Leitz incident fluorescent microscope. Appropriate controls were either sections receiving sera from healthy patients negative for salivary duct antibody, or sections receiving no human sera.

5.2.15 Cortisol Radioimmunoassay

Cortisol (Sigma, London) was measured in incubation media by a non-extraction radioimmunoassay method (Cortisol ^{125}I Radioimmunoassay Immunophase Kit, Corning Medical, Mass., U.S.A).



A



B

Figure 15

Reaction of the ductal cells of human submandibular gland (A) and mouse submandibular gland (B) using salivary duct antibody in an indirect immunofluorescence technique on frozen sections of the gland. A specifically ductal reaction is present.

A X460
B X776

Assays were prepared and counted in the Department of Steroid Biochemistry, Glasgow Royal Infirmary by Dr. M. Wallace. Between batch variation was below 8% and within batch was <5%. The antibody used has an 11% cross-reactivity with 11β hydroxycorticosterone, 12% with corticosterone and 10% with deoxycorticosterone. All other steroids tested, including cortisone, had a cross-reactivity of below 1%.

5.3 PLANIMETRIC AND STEREOLOGICAL ANALYSIS

5.3.1 Phase Microscopy and Photography

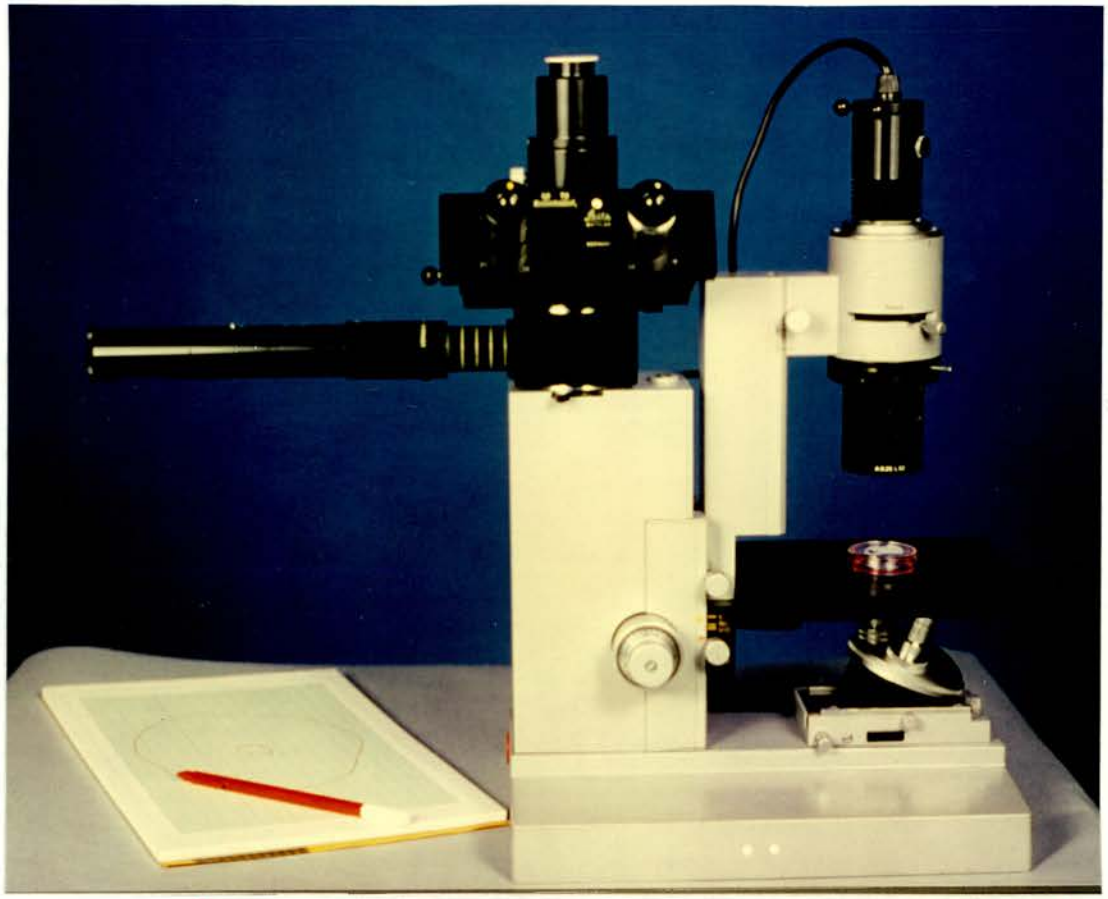
A Leitz inverted phase microscope was used to visualise unstained monolayers of cells. All photographs of living cultures were taken with phase contrast optics using a Leitz camera and Pan F film (Ilford Ltd., Ilford, Essex).

5.3.2 Planimetric Measurement of Culture Growth

Unlike monolayers from established cell lines, the outgrowth arising from a single explant if trypsinised to yield free cells for counting will also release cells from the explant. In addition, it is desirable to measure growth arising from each explant in a sequential manner. The required technique therefore has to be non-invasive, reproducible and relatively speedy, since prolonged removal of cultures from their gaseous environment is undesirable. Secondly, in measuring cellular outgrowth it would be advantageous not to rely on fixed and stained cultures since further investigation of cell origin by functional or immunological means may be required.

This is important for cultures of salivary gland as primary explants give no assurance of purity of cells present in the cellular outgrowth. This is inevitable since cultures originate from the mixed population of cells in a tissue explant.

A solution to the problem of growth measurement is to measure sequentially the area of cellular outgrowth by a drawing tube attachment (Figure 16a). Culture images were projected



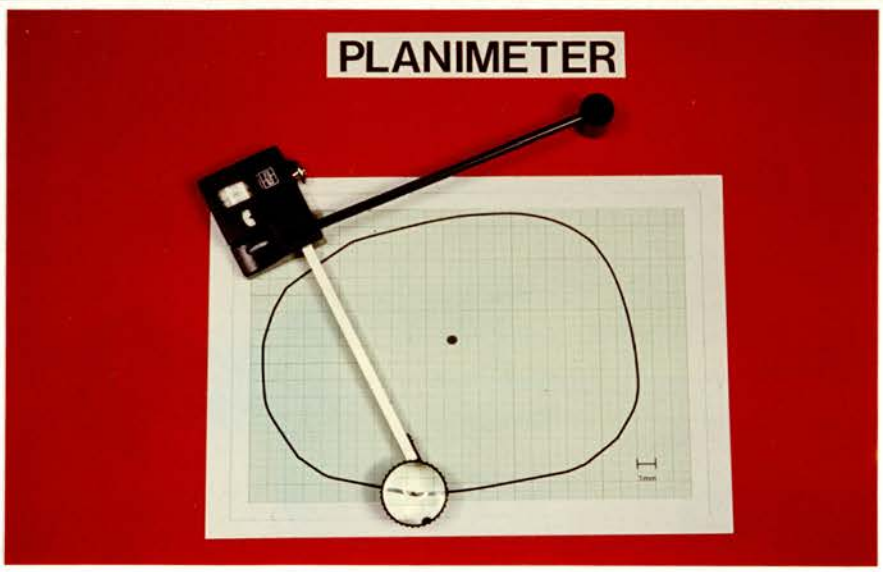


Figure 16

(a) Drawing tube attachment used to visualize growing culture and project the area of cellular outgrowth.

(b) Planimeter used to measure the projected cellular outgrowth.

on to paper and obliquely lit by an anglepoise light. The area of cellular outgrowth was drawn and measured by a planimeter (Figure 16b) as used for photographic prints (Meyer and Gerson, 1964 ; Barrington and Meyer, 1969).

The total cell area measured in square units at constant magnification can be deduced from the total cellular area by subtracting the area occupied by the explant. A standard 10x eyepiece lens was used throughout with a 6.4x inverted phase lens. Calibration of the area of cellular outgrowth was achieved using a 1 mm gridded slide from which 4 mm^2 = 550 $units^2$ of projected area.

The technique had the advantage of producing permanent records of the manner in which a cellular outgrowth was achieved whilst maintaining viability of the explant and the cellular outgrowth from it. Optically the system was satisfactory. Reproducibility of areas up to 100 $units^2$ was within 2%. Drawing was reasonably rapid, most areas being drawn in 3-4 minutes. This allowed cultures to be returned to the incubator before pH changes were visible in the phenol red. Changes in the area of cellular outgrowth achieved correlate most closely with changing cell numbers when cell size was constant and cell contacts within the cellular outgrowth were continuous.

5.3.3 Column Analysis of Cells

The planimetric method determines the area of cellular outgrowth produced but does not determine individual cells or cell populations within the outgrowth. For the latter purpose column analysis was used.

Coverglass cultures were fixed and stained with haematoxylin and eosin and mounted on a slide. The slide was placed on a Leitz Ortholux microscope (Figure 17a) and projected on to the screen of a teaching head (Figure 17b). Two vertical lines were drawn on the projection screen to define a column of cells of known width, the column passing

(a) (b)

Figure 17

(a) A Leitz Ortholux Microscope for counting cell columns.

(b) Teaching head of Ortholux Microscope which permitted cell size analysis within cell columns and classification of cells on a quantitative morphological basis.

through the explant. A second field was counted by defocussing and rotating the slide to align the certical lines at right angles to the first direction, again extending from the explant. This constitutes a systematic random sampling technique (Weibel, 1969). The overall magnification is x500 from cell to screen, being very adequate for the additional studies of individual cell morphology.

All the cells in a column stretching from the explant to the periphery were classified morphologically. Since cellular outgrowths from murine cultures are concentric around the explants and have continuous cell contacts these two columns are of approximately equal size. In 7 day cultures they may consist of several hundred cells.

Since a morphological definition of these cell populations is lacking they were classified as epithelioid or fibroblast-like cells by measuring the longest and shortest cell diameters. By this classification a cell is epithelioid if the shortest cell diameter is greater than two-thirds of the longest cell diameter, i.e. the cell is rounded. A fibroblast-like cell is defined as one in which the shortest diameter is less than one third of the longest cell diameter, i.e. the cell is spindle shaped. At the outset of this work it seemed this would be a useful quantitative index. Cell diameters were measured directly from the teaching head by dividers. The relationship between the two diameters was determined and each cell classified accordingly.

To determine, and to decrease the sampling error an accumulative means test (Chalkley, 1943) was applied to calculate the smallest sample required. These methods depend on the fact that as the size of the sample increases so the sampling error decreases (Chalkley, 1943 ; Weibel, 1969). Following an initial cell size count, the progressive mean for increasing number of counts is calculated with the data being plotted to show the accumulated mean values for the parameter against the number of points counted (Figure 18).

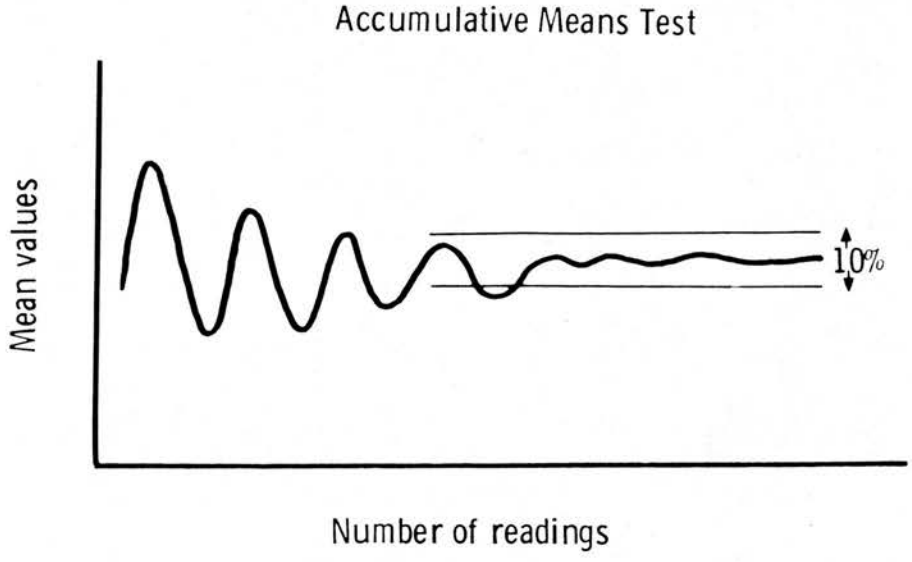


Figure 18

Operation of accumulative means test for determination of the minimum cell number required for statistical analysis.

For biological purposes a range of less than 10 per cent is acceptable and therefore the sample size required to achieve and remain within 10% of the final accumulative mean can be taken as the minimal sample size for that parameter.

In practice, the present morphological classification showed two clearly defined cell populations with less than 5 per cent of cells lying outwith this classification as shown in Chapter 6. This meant that cell numbers in columns were always greatly in excess of the required minimal sample sizes. Therefore, the selection of two columns for each culture was judged to be satisfactory for the present work.

5.3.4 Statistical Analysis

Comparisons between cultures to determine overall growth responses were made using standard statistical methods. All probabilities cited in the results were derived from standard tables. The results for attachment and growth of cultures were analysed using the Chi squared test. For neurotransmitter studies where the cell population response may exhibit kurtosis, non parametric analysis, Fisher's Exact probability test, was used. In addition to its use in the studies of cell morphology, the accumulative means test (Chalkley, 1943) was used to analyse the immunological and histochemical data to ascertain the number of cells in both categories required as a representative sample.

CHAPTER 6

OPTIMISING TISSUE CULTURE CONDITIONS FOR GROWTH OF MURINE SALIVARY GLANDS IN VITRO

6.1 RECOGNITION OF FACTORS AFFECTING CELL GROWTH IN VITRO

The problem of culturing developing tissue has long been recognised as difficult. Harrison (1908) made a detailed study of nerve fibres *in vitro* using frog lymph as a nutrient medium. Shortly afterwards Lewis and Lewis (1911 a, b) recognised the importance of the environment in maintaining cell viability. These authors predicted the introduction of defined synthetic media. The notion proposed by Claude Bernard (1878) that the environment reacted with the cell in a regulatory manner was beginning to be considered in the development of culture techniques. Progress into the production of defined media was made over the succeeding years as the role of small and large molecules in biological processes came to be recognised.

Media specifically for tissue culture work began to be produced by Earle (1943) and Hanks (1948). These media were developed to improve on "physiological salt solutions" (Tyrode, 1910) which in turn had been developed in an attempt to substitute simple salt solutions for serum or media. To date a large number of synthetic media have been developed both for long term and short term culture. A review article (Waymouth, 1965) cites 60 new chemically defined media as having been described between 1932 and 1962.

The diversity of defined media developed as a result of the realisation that many cells and many individual functions of cells require specific media (Evans, Bryant, Kerr and Schilling, 1964). Tissues, considered as cellular assemblies, may therefore require even more complex media. To study one cell parameter in a specific tissue from a given species of animal requires careful optimisation of the culture medium. Despite such optimisation it is generally recognised that problems of differentiation and further development of specialised cells in culture still remain.

6.1.1 Serum supplements

It has long been known that the addition of serum to a medium is required to produce optimal cell growth (Carrell, 1928 ; Vogelaar and Erlichman, 1933 ; Baker, 1936). "Growth stimulants" were considered to be present in serum and much effort went into isolating serum consistuents which alone would it was hoped, support growth (Pederson, 1944 ; Lucy, 1960 ; Holley and Kiernan, 1971). As the nature of these serum factors suggests, they were primarily considered to be involved in cell multiplication. Some cells do not require serum supplements in order to multiply (Evans, Bryant, Kerr and Schilling, 1964 ; Ham, 1965).

Detailed study of fibroblasts (Holley and Kiernan, 1971) has demonstrated that the final cell density achieved *in vitro* depends both on serum concentration and upon depletion of the serum by the cells. In addition, high cell density in untransformed cells greatly reduces growth (Bauer, 1977) and the saturation density at which growth ceases is characteristic of the cell type concerned (Stoker, 1967). The serum factors which permit such growth to saturation do show some species variability. Thus Holley and Kiernan (1971) demonstrated that on an equal volume basis rat serum was 2.5 times as active as calf serum for SV3T3 transformed mouse fibroblasts.

These observations demonstrate the need to define the ratio of, and origin of, the serum added to the basic medium. In the present study four sera from different species were investigated in a number of concentrations.

6.1.2 Cell growth autoregulatory factors

This is a complex problem. Under some circumstances cells in culture produce factors which regulate cell growth and division. In salivary gland, factors have been identified which alter growth *in vitro* but the relationship between, say, EGF release and the response of the secretor and adjacent cells remains undetermined. Such factors are doubtless present in

tissue culture sera. Thus calf endothelial cells synthesise certain globulins (Macarak, Kirby, Kirk and Kefalides, 1978) in the group of serum proteins known to have growth promoting activity (Paul, Lipton and Klinger, 1971). For some other cells, such as mouse fibroblasts, the secretion of low molecular weight protein with possible growth regulatory effects is transformation dependent (Gottesman, 1978). Transformed mouse fibroblasts also secrete a range of prostaglandins (Ritzi and Stylos, 1976) some of which stimulate epidermal growth in vivo (Bentley-Philips, Pauli-Jorgensen and Marks, 1977) and a number of murine and human cells in vitro (Schlammadinger, Zetterberg and Auer, 1976 ; Goldyne and Winkleman, 1976 ; Burstein, Gagnon, Hunter and Maudsley, 1977).

Whilst it is recognised these factors exist, experimentally the present work did not seek to establish whether or not salivary gland cell autoregulation occurs. Thus, the growth medium was investigated and defined using different base media combined with sera from different species. Other environmental influences which may alter growth, ambient temperature, humidity, and the composition of the gas phase were maintained constant.

6.1.3 Factors in relation to explant growth

In the terminology of the Tissue Culture Association (Federoff, 1967) an explant is described as "an excised fragment of a tissue or organ used to initiate in vitro culture". No definition of explant size is given and therefore the boundary between organ and primary explant culture is indistinct although the anticipated outcome of culture is of course different.

Trowell (1959, 1961) attempted to calculate the limiting radius of a tissue to permit tissue metabolism based on estimations of oxygen diffusion through a given tissue. Trowell (1961) concluded from his extensive studies that nutritional deficiencies were not responsible for the failure of many organs to survive for more than a few weeks in culture.

Embryonic tissues were more demanding. Easty (1970) also considered the problem of explant size and nutrient and oxygen diffusion and concluded that at least one dimension of the explant should not exceed 1-2 mm. Studies on primary explant cultures of murine salivary have used explants of diameter 0.5 - 1.0 mm (Wigley and Franks, 1976). Studies on organ culture of murine salivary gland aiming primarily to study culture survival do not give details of explant size (Tapp, 1967) but do draw attention to anoxia in central cells in the culture. This anoxic phenomenon has been likened to the hypoxic changes observed in salivary duct cells in vivo following duct ligation (Junquiera, 1951). In the present study a range of salivary gland explants were prepared and the relationship between explant size and both adhesion of the explant and the ability to permit cellular outgrowth was assessed.

Stability of the tissue culture milieu has only comparatively recently been studied in primary cell culture. Detailed studies by McLimans, Crouse, Tunnah and Moore (1968) of oxygen and gas diffusion in explant systems have enabled calculations to be made relating diffusion of oxygen in the culture fluid. A controlled environmental system is essential if cell function and growth are to be studied in a tissue in vitro. The importance of CO_2 as a potential regulator of cell metabolism has long been known (Lille, 1909 ; Swim and Parker, 1958 ; Longmore, 1966 ; Kieler and Gromek, 1967). For this study a constant humidity system delivering 5% CO_2 in 95% air was used. The growth of many mammalian cells is critically temperature dependent (Williams, Rittenhouse, Iwata and Fox, 1977), and pH dependent. The culture temperature was maintained at 37°C.

Medium pH was also kept constant by appropriate buffers, it being found during the study that salivary gland cultures were not marked acid producers. This data is not presented in this thesis.

Cell environment is not only the liquid-phase, gas tension and temperature presented to the cell. The surface to which the cell is adherent and the proximity and number of adjacent cells are other important considerations.

The substratum is known to modify cell behaviour (Maceira-Coelho and Avrameas, 1972). Moreover various factors, principally high molecular weight glycoproteins are thought to be required for cell adhesion. For some cells this glycoprotein has been partially characterised and called Cell Adhesion Factor (CAF) (Pearlstein, 1976, 1978 ; Pearlstein and Gold, 1978). Collagen is also involved in cell attachment and may interact with CAF (Klebe, 1974) but protein synthesis is not required for cell attachment (Klebe, 1975).

When cultured directly on artificial substrata cells have been described as synthesising substrate - attached material (SAM)[+] which is a heterogeneous mixture of proteins and polysaccharides (Culp and Black, 1972). In contrast to CAF mediated adhesion which may relate to pre-existing pools of CAF in the cell, SAM is actively synthesised as determined by incorporation of radioactive precursors of proteins and polysaccharides (Terry and Culp, 1974). Serum derived factors which coat cells may also be important in cell attachment (Culp and Buniel, 1976 ; Stamatoglou, 1977 ; McKeehan and Ham, 1976). Another relevant observation concerning substrata is that cell shape is involved in growth control (Folkman and Moscona, 1978). Cell shape can partly be determined by adhesiveness to substratum.

In the present study six substrata were investigated both for ability to produce explant adhesion and permit cellular outgrowth. The ease with which the tissue could be prepared for ultrastructural studies on cell attachment to substratum and cell to cell attachment was also noted.

[+] The abbreviation used here is for this material and does not refer to S-adenosyl-methionine which by convention is also SAM.

This summary outlines the variables which operate during *in vitro* cellular studies. The systematic analysis of each factor is now presented in an attempt to define precisely the normal range of growth of murine salivary glands. This enables the effect of selected neurotransmitters on such growth later to be established more precisely. The techniques used have been presented in Chapter 5.

6.2 EXPERIMENTAL FINDINGS

6.2.1 Variation in explant size

Following removal of the submandibular salivary glands from the newborn animal, disaggregation of the tissue was by physical means. Tissues were carefully manipulated to minimise trauma. Fine curved scissors were used to finely chop the tissue whilst keeping it moist (Parker, 1961). Other methods of tissue dissociation (Sykes, Whitescarver, Briggs and Anson, 1970 ; Amsterdam and Jamieson, 1972 ; Matsumura, Yamanaka, Hashizume, Irie and Nitta, 1975) were investigated and found unsatisfactory for the present study since they did not produce the purity of cell populations claimed. The mechanical disaggregation procedure is traumatic to tissues but does circumvent the problems of cell selection using trypsin (Hodges, Livingston and Franks, 1973) or protease (Burger, 1970).

Medium 199 with 20 per cent newborn-calf serum was supplemented with penicillin and streptomycin. Glass was the chosen substratum amd 30 mm^2 petri dishes were chosen containing an Alconox cleaned glass coverslip (22 x 22 mm) (Chance, England). Four explants were placed in each petri dish and 2 millilitres of growth medium added. Growth media were changed on day 3 and cultures examined by inverted phase microscopy on day 7 for evidence of cellular outgrowth. Table 1 shows that for practical purposes cellular outgrowths resulted from all explants initially in the range 0.2 to 0.4 mm in diameter (to nearest 0.01 mm). This explant diameter was adopted for all future studies on murine salivary gland growth.

TABLE 1

Growth of murine salivary cells

on different substrata

	Substratum	Mean Area (units)^2*	Explants Attached as percentage Initiated
A	Alconox washed glass	320	80 (n = 28)
B	Carbon coated glass	20	50 (n = 30)
C	Poly-l-lysine coated glass	80	58 (n = 26)
D	Thermanox	400	80 (n = 28)
E	Melinex	300	58 (n = 26)

*

Units : 550 units^2 = 4 mm^2

Conditions are described in the text.

Adhesion success rates of 80% were achieved for explants from newborn animals and 70% for explants from adult animals after 7 days in culture. There was no correlation between explant size and adhesion ability.

6.2.2 Explant adhesion

Much of the work on tissue culture is concerned with initiating culture growth from discrete fragments of tissue. For some tissues, but not all (New, 1974) it is advantageous to secure the tissue to the glass until tissue outgrowth anchors the fragments to the chosen surface. That this is a problem with *in vitro* adult mouse submandibular salivary gland culture has already been reported (Wigley and Franks, 1976).

Data from the study of explant size shows that explant adhesion is not 100%. A figure approaching this would minimise tissue waste. Therefore the following study aimed to determine whether explant adhesion could be improved using cyanoacrylate adhesion without deleteriously affecting cellular outgrowth.

Various methods of achieving adhesion of explants are reported in the literature. These include holding the explant in place with a thin film of cellophane (Rose, Pomerat, Shindler and Trummell, 1958) or by allowing the explant to dry onto the surface prior to addition of medium (Levi-Montalcini and Seshan, 1973). However, neither of these studies has involved salivary glands.

The initial treatment of the salivary tissue was as for the explant size studies. Submandibular salivary glands from 15 male neonatal CFLP mice were separated from the adjacent sublingual and parotid glands along fascial planes with the aid of a dissecting microscope and removed aseptically. Five adult male CFLP mice were treated similarly. Following incubation of the entire gland at 37°C in transport medium (199 containing penicillin, streptomycin and mycostatin) for twenty minutes the glands were cut with fine curved scissors to produce explants with a largest diameter of not more than 0.4 mm.

Polystyrene surfaces dissolve in the solvents involved in the dehydration steps of routine staining procedures and therefore the surface to which adhesion was required was glass.

Thirty millimetre petri dishes containing sterile glass coverslips (22 x 22 mm) were prepared with each containing 2.0 ml of 20 per cent newborn-calf serum in 199 medium : the sterile cover glasses had previously been washed in Alconox. Four explants of uniform size were placed on each coverslip immediately before addition of the medium. In two separate experiments forty-eight explants were used for each trial of direct culture alone and forty-eight explants for each of the two cyanoacrylate compounds which were Permabond 102 (Staident Products Limited, England), main constituent ethyl alpha cyanoacrylate, and Loctite (Loctite U.K., England), main constituent type 3-ethyl cyanoacrylate. It was found that storage conditions and limited shelf life of the cyanoacrylate adhesives produced variable adhesion results. Fresh adhesive stored at 4°C was therefore used. In addition twenty-four explants were bonded with methyl methacrylate ultraviolet light (wave-length 366 mm) activated materials, (Nuvaseal ; Amalgamated Dental Company, England), and cultures exposed to ultraviolet light alone for the one minute needed for Nuvaseal activation.

All dishes were kept at 37° in a humidified atmosphere of 5% CO_2, 95% air and the experiment stopped four days later. Preliminary studies had shown that all cellular outgrowths from newborn submandibular gland tissue had already occurred by two days, and for the adult tissue by three days.

Adhesion of explant was considered successful if after four days it was still attached to its original position and not freely mobile. Reattachment of explants which became detached following addition of medium was not observed. Growth was considered to have occurred if there was cellular outgrowth from the explant.

The results for attachment and growth were analysed statistically using the Chi squared test for significance and summarised in Tables 2 and 3.

TABLE 2

The proportion of growth and failure of attachment in newborn mouse submandibular gland explants using different adherents in vitro

Group	Adherent	Explants Attached at day 7	Explants growing at day 7 following attachment	Overall growth at day 7 (Irrespective of attachment)
A	Direct culture	39/48 (81%)	15/39 (38%)	15/48 (31%)
B	Nuvaseal	18/24 (75%)	0/18 (0%)	0/24 (0%)
C	U.V. light alone	11/24 (46%)	3/11 (27%)	3/24 (13%)
D	Permabond	44/48 (92%)	15/44 (34%)	15/48 (31%)
E	Loctite	44/48 (92%)	2/44 (5%)	2/48 (4%)
	Analysis	A:C $p<0.01$	A:E $p<0.001$ D:E $p<0.01$	A:C $p<0.1$ A:E $p<0.001$ D:E $p<0.001$

Cultures were grown in medium 199 containing 20 per cent (v/v) newborn calf serum as described in the text.

TABLE 3

Attachment and growth achieved by direct culture and by cyanoacrylate-aided adhesion of adult and newborn mouse submandibular gland explants in vitro

Group	Tissue	Adherent	Explants Attached	Explants Growing Following Attachment	Overall Growth (Irrespective of Attachment)
A	Adult	Direct culture	31/48 (65%)	6/31 (19%)	6/48 (13%)
B	Adult	Cyanoacrylate	43/48 (90%)	13/43 (30%)	13/48 (27%)
C	Newborn	Direct culture	39/48 (81%)	15/39 (38%)	15/48 (31%)
D	Newborn	Cyanoacrylate	44/48 (92%)	15/44 (34%)	15/48 (31%)
Analysis			A:B $p<0.1$ A:C $p<0.1$	A:C $p<0.1$	A:B $p<0.1$ A:C $p<0.05$

Cultures were grown in medium 199 containing 20 per cent (v/v) newborn calf serum as described in the text.

Statistically identical adhesion values for CFLP neonatal male mouse submandibular gland explants were obtained with direct culture on glass alone and with both cyanoacrylate compounds (Permabond and Loctite). Permabond allows growth comparable to that obtained with direct culture alone indicating toxic diffusion products in the vicinity of the explant are not a problem : Loctite by comparison greatly reduces growth. This is apparently not prejudicial to the efficacy of these cyanoacrylate compounds in a variety of in vivo situations including ophthalmology (Faulborn, 1976 ; Regenbogen, Romano, Zuckerman and Stein, 1976), urology (Kelami, 1976), surgery (Goldin, 1976) and neurosurgery (Chou, 1977).

Ultraviolet light, 366 nm, alone reduces adhesion but statistically fails to inhibit growth (Table 2). Nuvaseal produced greater adhesion than ultraviolet light alone but growth was inhibited (Table 2). This may have been due to a combination of the physical effect of ultraviolet irradiation and free radical release following ultraviolet activation of the cement. It is possible that the local environment around the explant may have been altered so as not to be conducive to producing cellular outgrowth but the effect on attachment and how this relates to cell viability is unclear.

There is no significant difference in attachment of newborn and adult tissues using direct culture (Table 3,a).

Permabond does increase the attachment rate of adult salivary glands and since it does not decrease the growth it has an overall effect of increasing the growth yield.

The results also illustrate that in situations where a newborn tissue explant itself is capable of adhesion neither of the bonding cyanoacrylates used here will apparently improve growth and adhesion. In situations where explant adhesion or organ adhesion is difficult to achieve, Permabond appears to permit growth equivalent to that which would occur were unaided adhesion possible. When adult salivary gland explants are used Permabond improves adhesion compared with that during direct culture increasing the yield of growing explants of newborn and adult tissues.

Permabond was routinely used in the early phases of this study where cell growth was being studied macroscopically. Later, in connection with histochemical and electron microscopy analyses it was found that Permabond interfered with these techniques and so its use in this connection was abandoned.

6.2.3 Substrata

The complex interrelationship between cell and substrata has been mentioned. The following substrata were studied for their ability to permit optimal growth :

a) Alconox cleaned glass coverslips
b) Carbon coated glass coverslips
c) Poly-l-lysine coated glass coverslips
d) Thermanox (polycarbonate)
e) Melinex (polyethyleneterrphthalate)

The growth medium was 199 containing 20 per cent (v/v) newborn calf serum and standard concentrations of penicillin and streptomycin.

Using routine dissection procedures, explants of newborn mouse submandibular gland of less than 0.4 mm largest diameter were prepared. Four explants of 0.4 mm largest diameter were placed on each substratum and incubated for 7 days with the medium renewed on day 3. On day 7 any cellular outgrowth which had occurred was measured planimetrically. All cultures were then prepared for transmission electron microscopy using an epoxy embedding method of Nopanitaya et al. (1977) and the ease with which each substrate could be processed, was assessed.

Adhesion success rates and the area of cellular outgrowth achieved by neonatal submandibular salivary gland explants on each substratum are summarised in Table 1. The use of Thermanox as a substratum permits maximum cellular outgrowth but this substratum is impractical in circumstances where exposure to the alcohol dehydration steps following staining procedures is necessary. Direct culture on glass surfaces required chemical dissolution with hydrofluoric acid to remove the Araldite embedded culture. Although coating the glass with

carbon or poly-l-lysine permitted its physical separation with Araldite, cell growth was seriously impaired.

Accordingly, for studies on the cell-to-cell contacts present in salivary gland cells in vitro and cell-to-substrata adhesion, cultures were grown routinely on Alconox treated glass coverslips which were processed for ultrastructural studies as in the methods section.

6.2.4 Culture media

Parotid, sublingual and submandibular salivary glands were aseptically removed from male newborn CFLP mice. Explants of 0.4 mm largest diameter were cultured 4 per coverslip on Alconox cleaned glass coverslips. Two millilitres of growth medium was added and renewed on day 3.

The following five media (all Gibco Biocult) were evaluated for promoting salivary gland growth : 199 (Earle's modified salts, L-glutamine and 15 mmol/l NaHCO3), MEM (Earle's salts and L-glutamine), William's (medium D), Trowell's (medium T8) and Waymouth's (medium MB752/1). In these initial trials, an arbitrary supplement of 20% newborn calf serum with penicillin and streptomycin was used. Planimetric and column analysis techniques were used to quantify growth achieved by day 7.

Results of the range of growth achieved for newborn submandibular salivary gland is shown in Figure 19 . Comparable results were achieved for sublingual and parotid glands. More complete results for all media on the three major salivary glands of the newborn mouse are listed on Tables 4 and 5. At 7 days all results between individual glands in different growth media are highly significant except between 199 media and William's media which show no difference for growth of male newborn mouse sublingual glands. An additional observation was that the choice of medium affected the ability of newborn calf serum to permit cell growth and division but did not influence explant attachment to the substratum. Feldman and Wong (1977) found that the choice of sera greatly altered cell

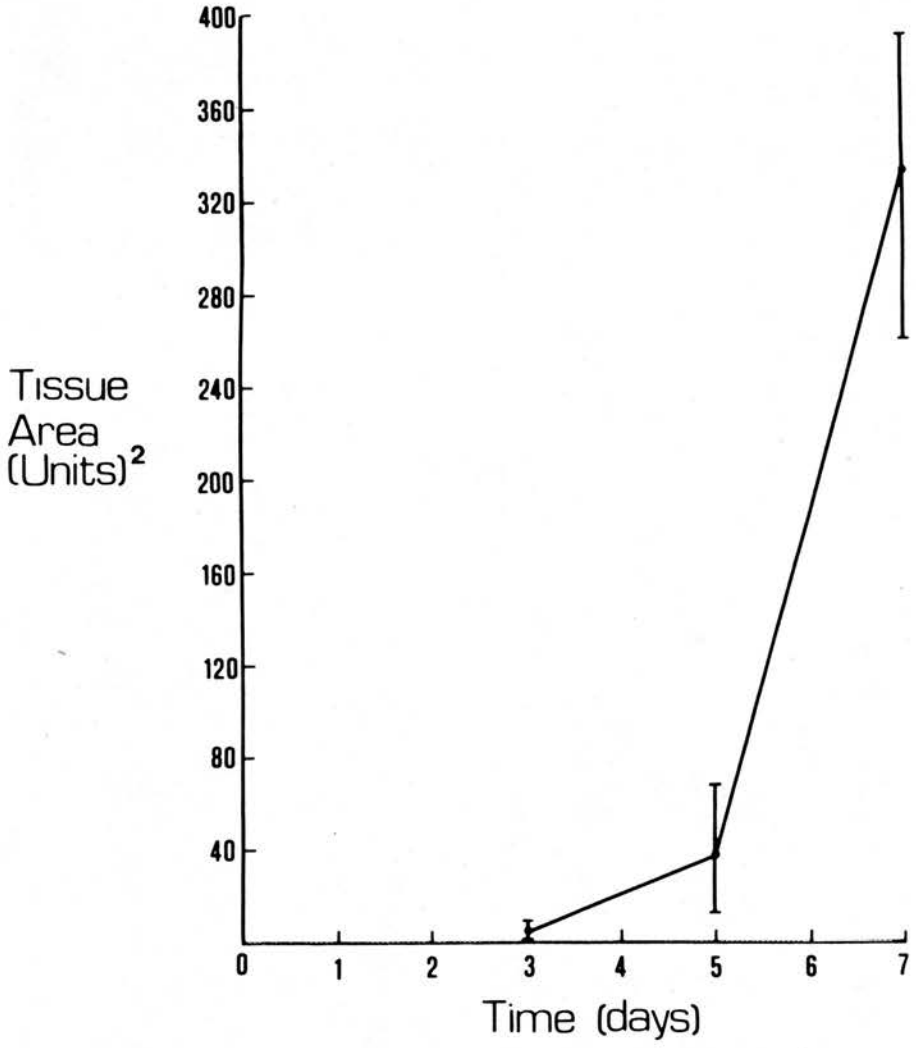


Figure 19

Growth of newborn mouse submandibular salivary gland cultures on glass in medium 199 with 20% (v/v) newborn calf serum. Bars represent one standard deviation.

Tissue area units : 550 units^2 = 4mm^2

TABLE 4

Mouse salivary gland explant growth in different media supplemented with 20% newborn calf serum

Medium	Tissue	Number Observations	Tissue Area (units) 3 day	5 day	7 day
199	SM (submandibular)	44	5.35	38.6	331.4
	SL (sublingual)	31	9.2	39.4	238.1
	P (Parotid)	44	14.8	70.05	341.2
Williams	SM	39	11.0	66.2	134.2
	SL	31	4.16	66.6	225.2
	P	12	5.84	15.8	53.1
Waymouth	SM	37	No growth	No growth	No growth
	SL	49	No growth	No growth	No growth
	P	31	No growth	No growth	No growth
Trowells	SM	48	0.6	6.69	8.5
	SL	39	1.58	8.84	6.1
	P	23	No growth	No growth	No growth
MEM	SM	38	6.27	13.33	32.5
	SL	37	2.62	9.34	19.9
	P	34	10.10	68.7	237.0

Explants were grown on Alconox-treated glass coverslips.
Tissue area units : 550 units^2 = 4 mm^2.

TABLE 5

Analysis by anatomical site of
mouse salivary gland growth in different media

	SM Submandibular	SL Sublingual	P Parotid
199 v Williams	$p<0.001$	$p<0.02$	$p<0.001$
199 v Trowells	$p<0.001$	$p<0.001$	NP
199 v MEM	$p<0.001$	$p<0.001$	$p<0.001$
Williams v Trowells	$p<0.001$	$p<0.001$	NP
Williams v MEM	$p<0.001$	$p<0.001$	$p<0.001$
Trowells v MEM	$p<0.001$	$p<0.001$	NP

NP = not performed

Data taken from Table 4 has been analysed
by a Chi squared method.

attachment in primary cultures. Gibco Waymouth medium produced no growth in this series of experiments and no other source was used.

Different media thus affect the ability of serum to permit cell growth and division but without apparently influencing explant attachment to the substratum.

6.2.5 Species of origin of sera

Holley and Kiernan (1971) noted the differing growth promoting ability of serum from different species.

Sera tested in this study of neonatal gland cultures were : newborn calf serum, foetal calf serum, horse serum (all Gibco Biocult) and mouse serum. The mouse serum was obtained by bleeding 6 week old male CFLP mice. Each serum was used at a concentration of 20 per cent (v/v) in medium.

Three separate trials were conducted using pooled sera from three separate batches to reduce possible inter-batch variation.

Growth achieved in 20 per cent (v/v) newborn calf, adult mouse and adult horse sera after 7 days is illustrated in Figure 20. Foetal calf serum does not appear on this figure as it rather surprisingly, but reproducibly, did not support explants in producing a cellular outgrowth. Use of adult mouse and adult horse sera was discontinued.

Medium 199 with 20 per cent newborn calf serum was used routinely for subsequent cultures. Little inter-batch variation between sera was observed.

6.2.6 Concentration of serum

Of the sera tested, newborn calf sera produced optimal growth but had been only used arbitrarily in a 20 per cent concentration. In further studies concentrations of 10, 20 and 30% (v/v) serum in medium 199 were assessed for support of cellular outgrowth .

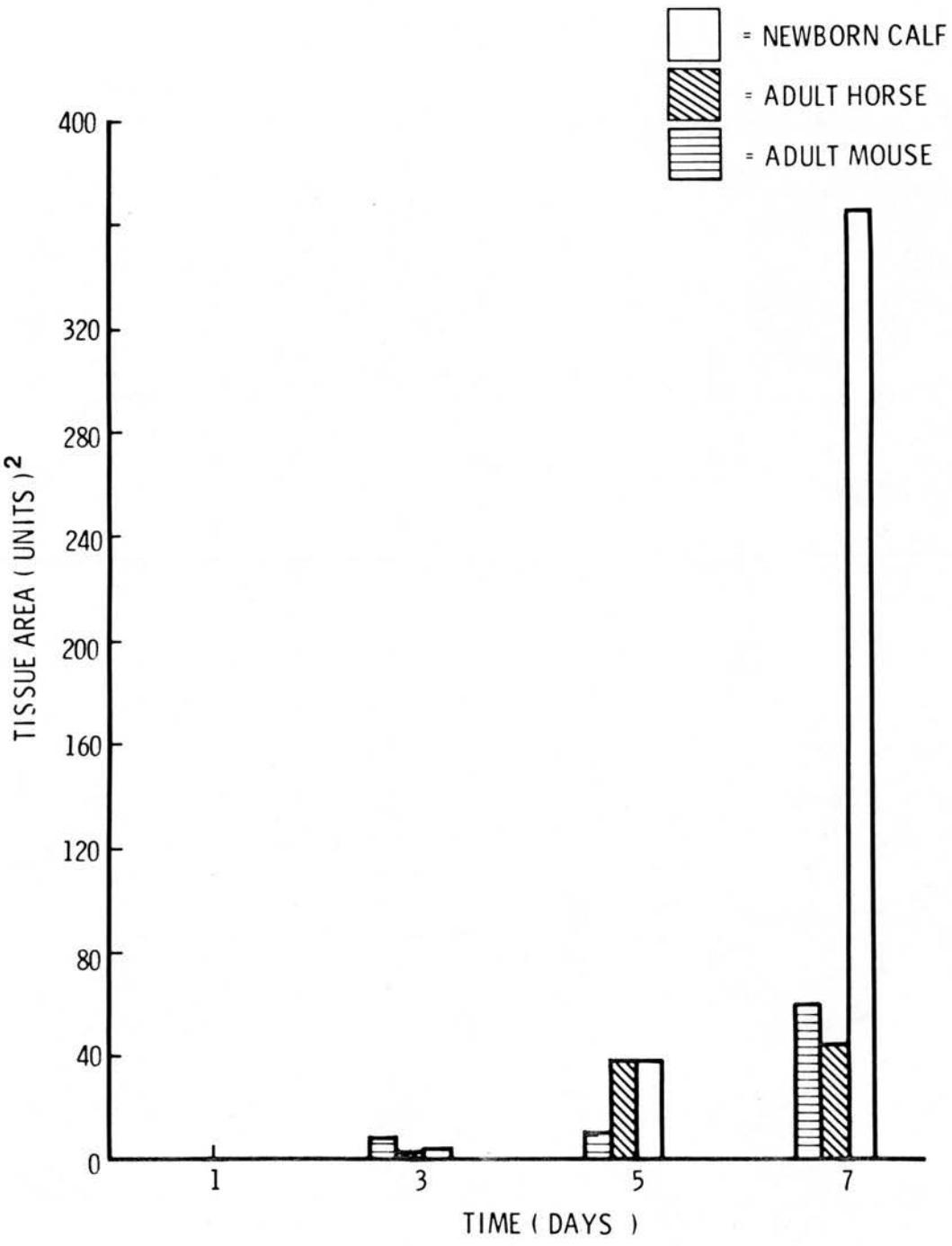


Figure 20

Histogram showing growth in medium 199 supplemented with 20% (v/v) newborn calf serum, adult horse and adult mouse serum. Newborn mouse submandibular salivary glands were grown on coverglasses as described in the text from day 0.

Tissue area units : 550 units^2 = 4mm^2

Newborn male submandibular salivary glands were prepared as previously using the now standard techniques of explant size, 199 medium and glass substrate. Medium was changed on day 3 and growth analysed by planimetry on day 7. Results are presented in Figure 21.

The results demonstrate that the hitherto fortuitously chosen 20 per cent (v/v) concentration of newborn calf serum in 199 medium is optimal. This differs from Holley and Kiernan's (1971) results in which fibroblast response to 10 per cent, 20 per cent and 30 per cent newborn calf serum linearly increased over this range although cell density in their experiments was comparatively low.

6.2.7 Conclusions

Culture of the salivary gland explants produced either cellular outgrowth and the explant assumed a spherical form or alternatively no cellular outgrowth occurred and in this case the explant was of a flat appearance such as is seen in Figure 22. These flat explants maintained viability with residual ductal and acinar architecture persisting : this pattern of differentiation was never observed in explants producing a cellular outgrowth.

It is a common observation that culture on plastic surfaces frequently produces growth greater than that achieved on glass. This qualitative finding has been substantiated for salivary explant cultures on a series of substrata. However, it is known that complex inter-relationships exist between the ability of cells to adhere to the substratum and to the influence of serum growth factors, which govern cell shape and growth control (Feldman and Wong, 1977 ; Greenberg, Grove and Cristofalo, 1977 ; Folkman and Moscona, 1978).

The application of increasingly defined media to cell and organ culture systems and the continuing developments in the composition of these media have been extensively reviewed (Ham, 1974 ; Paul, 1975 ; Gospodarowicz and Moran, 1976).

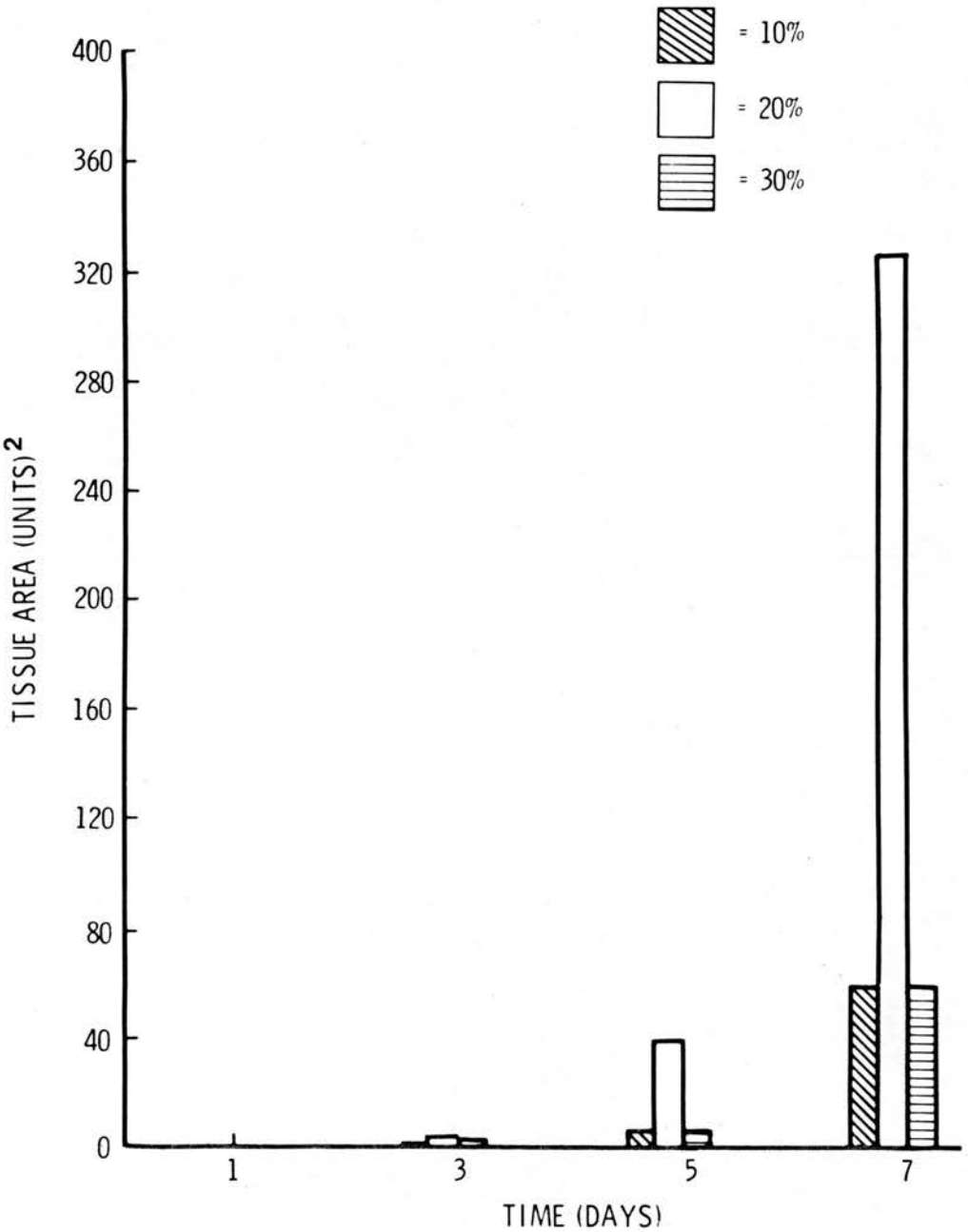


Figure 21

Histogram demonstrating increasing growth of newborn mouse submandibular gland in 20% newborn calf sera supplemented medium 199.

Tissue area units : 550 units² = 4 mm²

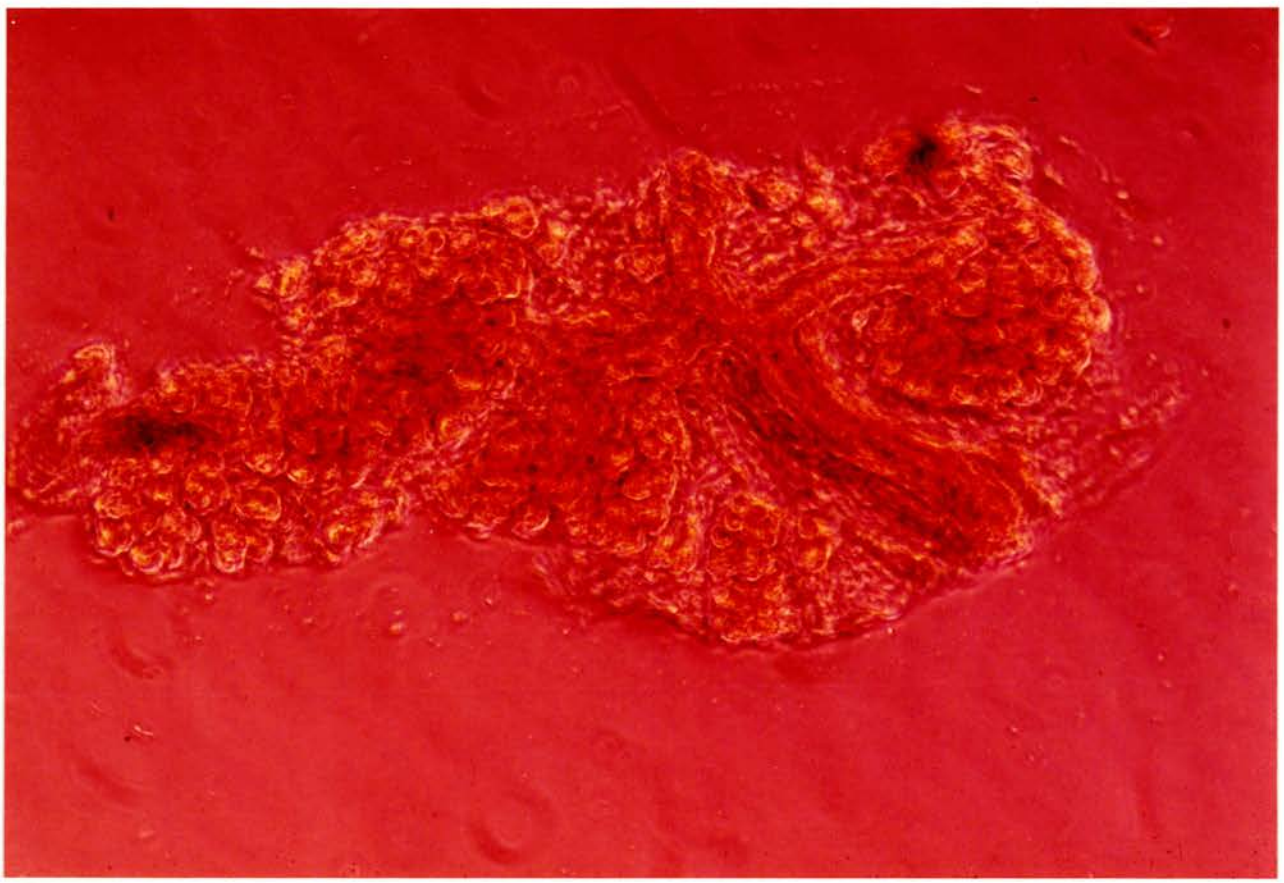


Figure 22

An example of the flat organotypic culture of newborn mouse submandibular gland. A ductal pattern is discernible with surrounding lobular, presumably acinar, cells.

X 100

The choice of medium affects the ability of serum to permit salivary cell proliferation whilst not apparently influencing attachment to the substratum. The statistically significant differences in cellular outgrowth from explants from the same gland receiving the same serum supplement but in different basal media point to a fundamental lack in our understanding of the nutritional requirements of these salivary cells.

The growth promoting activity of serum has been reported as showing little species specificity (Clarke and Stoker, 1971). In contrast, the present study indicates that there are substantial inter-species variations in the growth promoting property of serum. Further there appears to be an optimal serum concentration of 20 per cent which if exceeded resulted in growth inhibition.

Definition of which components of serum are specifically required for proliferation has yet to be achieved but the functions of serum appear to be stimulation of cell growth and division in addition to influencing cellular attachment to the substratum (Culp and Buniel, 1976 ; Feldman and Wong, 1977).

CHAPTER 7

IDENTIFICATION OF EPITHELIAL CELL POPULATIONS IN MURINE SALIVARY GLAND CULTURES

Primary explant culture of adult rodent salivary glands inevitably produces a mixed cell population. Fundamental problems which remain are to determine the origin of the different cells and to study their role in differentiation. Since culture of non-transformed epithelial cells alone has frequently been difficult to achieve, an alternative approach is to accept the non-homogeneity of a culture and attempt to identify specific epithelial cell populations present.

The control of tissue behaviour is best studied when some degree of differentiation has been initiated. However, foetal tissue is, in general, more successfully cultured (Trowell, 1959). The salivary glands of the neonatal mouse where the acini are just developing from the ductal epithelium (Yohro, 1970) are a useful source of partly differentiated tissue.

There are at least four types of cells in the adult salivary gland. This integrated structural and functional character is clearly not readily preserved in monolayer culture. For example does each acinar cell require several juxtaposed myoepithelial cells ; or is a mixture of myoepithelial and fibroblast-like cells sufficient to maintain ductal epithelial cell integrity ? Alternatively acinar or ductal cells may be capable of existing independently in monolayer culture.

It is to be expected that the dynamics of growth from an explant will therefore be complicated. In addition it will be necessary to identify each contributing cell and hence to decide upon the most reliable way of making the necessary distinctions.

By identifying epithelial cell populations within the outgrowth, the role of chemical mediators upon both salivary gland proliferation and differentiation may subsequently be studied. For studying the origin of cells in the outgrowth, a combination of light and electron microscopy (Sect. 7.1 to 7.3), autoradiography (Sect. 7.4.1) and histochemistry (Sect. 7.4.2 and 7.4.3) was used in the present study.

7.1 LIGHT MICROSCOPY OF SALIVARY GLAND CELLS AN AN INDEX OF THEIR ORIGIN

To determine whether cell morphology at the light microscopic level would be of value in establishing cell origin in the cellular outgrowth, photographs of all cultures both in the growing and in the stained state were examined. Growing cultures were photographed using a Leitz phase microscope. In addition, cultures were rinsed in phosphate buffered saline, fixed in 10% formal saline and stained with haematoxylin and eosin.

Figure 23 demonstrates the *in vitro* cell morphology of newborn mouse submandibular gland, compared with mouse fibroblasts NCTC 929, human lamina propria and HeLa cells. It is apparent that even within cell lines (HeLa and NCTC 929) cultured *in vitro* two dimensional cell morphology is variable. The area of cell attachment will produce differing two-dimensional morphologies but this is doubtless not the only determinant. Important aspects, namely cell division, differentiation and cytoskeletal structure are poorly understood as determinants of cell morphology. As in other cell culture systems, salivary gland cells which attach poorly tend to appear rounded.

In order to investigate the relationship between cell morphology and these other criteria, an arbitrary division of cell populations was made, since quantitative criteria for identifying salivary gland cell populations morphologically is lacking.

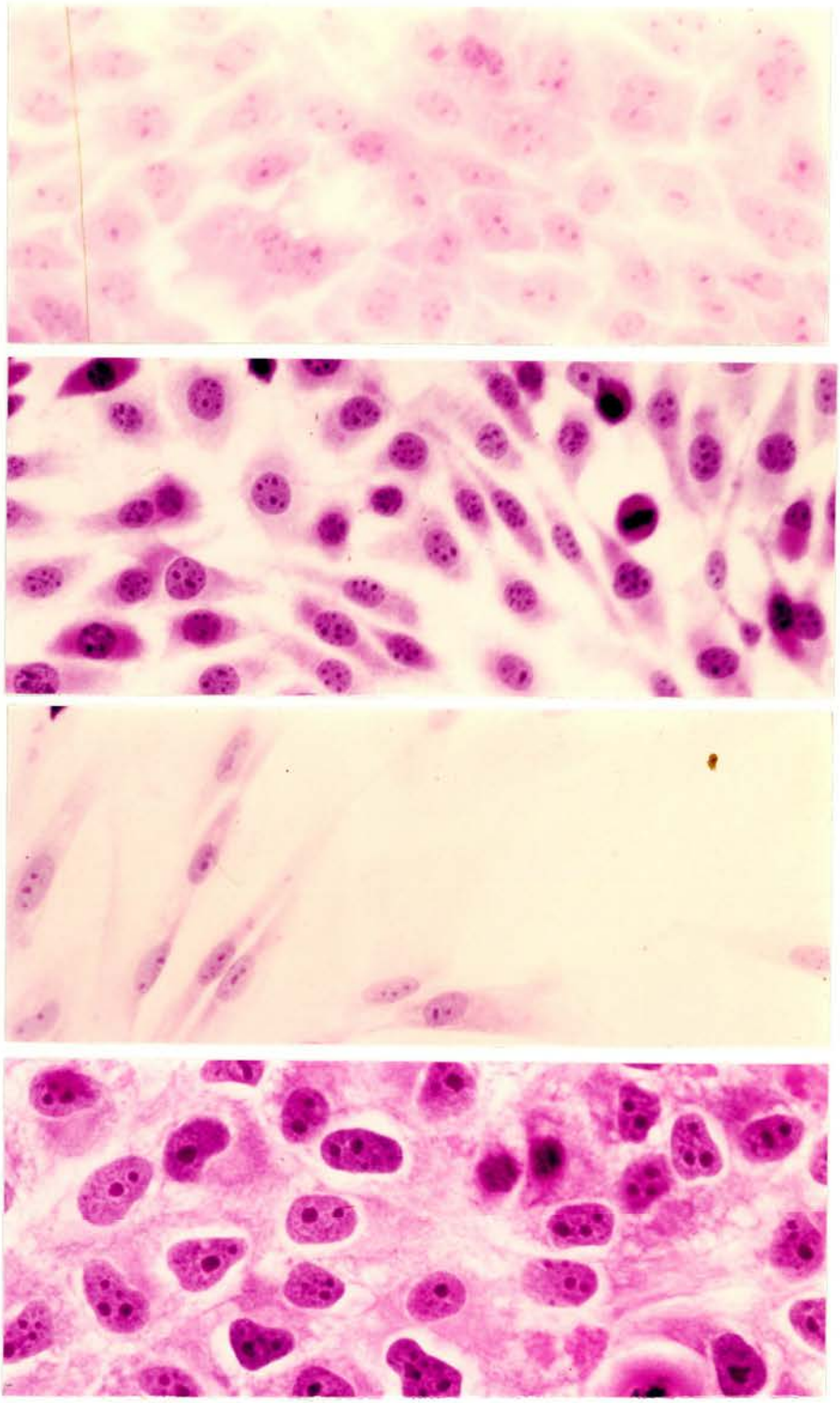


Figure 23

Comparative *in vitro* cell morphology of newborn mouse submandibular gland, mouse fibroblasts (NCTC 929), human lamina propria and Hela cells. Clearly *in vitro* two dimensional cell morphology is variable even within established cell lines (Hela and NCTC 929) and cell morphology alone is not a good index of cell origin.

X 400

Epithelial cells, epithelial-like cells, fibroblasts and fibroblast-like cells are relatively loosely defined (Federoff, 1967). This definition is adhered to here ; i.e. fibroblasts are those cells which result in extracellular fibre formation whilst epithelial-like cells yield continuous mosaic-like sheets. For practical purposes this definition is complex for EM techniques must be used to detect extracellular fibre synthesis unless specific antibody is available.

Clearly to use these techniques on every explant and cell culture would be too time consuming, so an arbitrary quantitative classification of cell type has been introduced and used in this study. The distinction into cell types is on the basis of measurement of the longest and the shortest cell diameter, as described in the Methods Section. No absolute classification into cell origin is implied by this classification. Later studies however such as ultrastructural studies may demonstrate collagen formation and a fibroblast-like cell would be then considered a fibroblast. Similarly the demonstration of a specific ductal reaction histochemically or immunologically would result in an epithelial-like cell being considered ductal epithelial in type.

This working classification is simple and has been valuable in classifying large numbers of cells quickly and quantitatively and, secondly, for demonstrating the relationship between cell morphology and specific criteria of cell origin.

In Figure 24 the largest cell dimension is analysed in terms of the distribution of cells in a mixed cell population, allowing the cell distribution to be readily distinguished. By this method the cell populations present in the cellular outgrowth represented in Figure 24 are found to consist of two morphologically distinct cell populations. The group which, by the present classification, referred to as epithelioid have a largest cell diameter of 57 μm. In many populations analysed during this work less than 5% of cells lay outwith this arbitrarily-defined morphological classification.

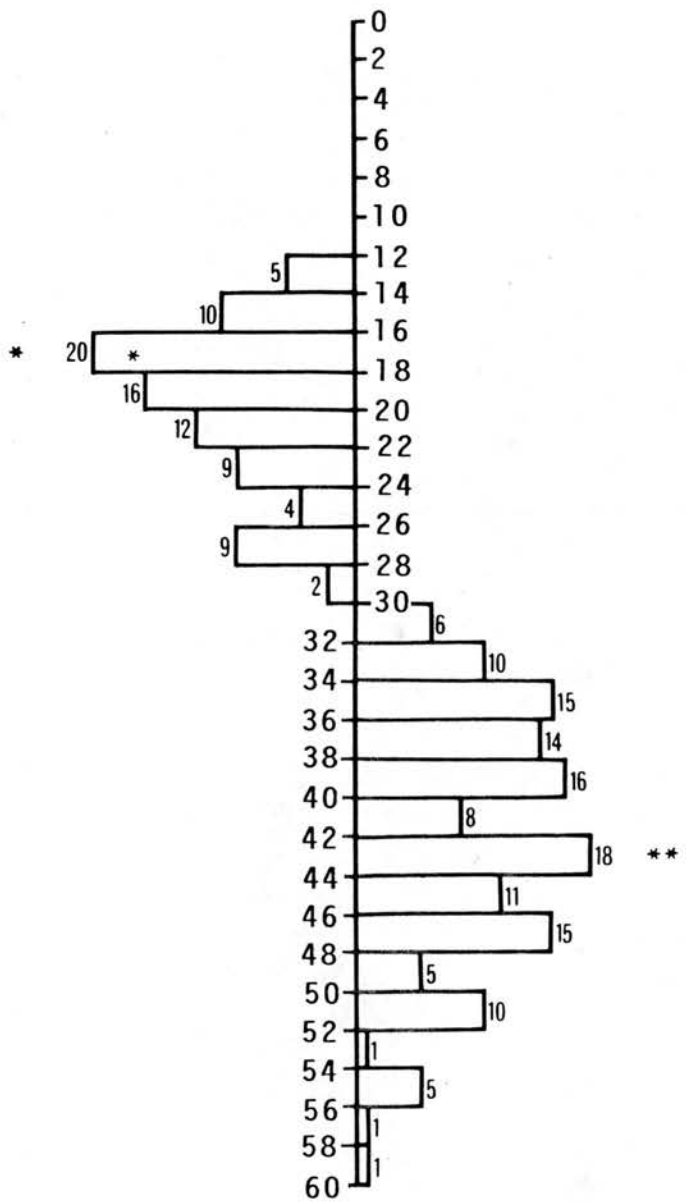


Figure 24

Analysis of cell populations by largest cell diameters in cells growing from newborn mouse submandibular gland explants. Two morphologically distinct cell populations are present, one designated epithelioid* the other fibroblast-like**. This classification is of value when comparing cell morphology with absolute indices of cell origin but it itself does not imply a given cell origin for either cell type. Vertical scale in microns.

Epithelioid cells were scattered either singly or in groups throughout the cellular outgrowth and showed no statistically significant predilection for the region immediately adjacent to the explant.

There were no other useful identifying features at the light microscopic level. The epithelioid cells from the mouse cultures had an oval nucleus and several distinct nucleoli. No obvious granules were present in the cytoplasm although vacuoles were occasionally noted. The fibroblast-like cells also had oval nuclei but these cells had characteristic cytoplasmic extensions. There was no evidence of myoepithelial cells as previously described in rat salivary gland cultures (Konisberg, 1960 ; Cutler and Chaudhry, 1973 a).

7.2 ULTRASTRUCTURE AND THE ORIGIN OF SALIVARY GLAND CELLS

At the outset ultrastructural studies were aimed at determining cell origin by means of an investigation of cell-to-cell contact and an examination of cell adhesion to the different culture substrata.

7.2.1 Cell-to-Cell Contact

Cell-to-cell contacts provides one means of investigating cell origin. Junctions specific to epithelially derived cells (Farquhar and Palade, 1963) or found on endothelial cells (Yee and Revel, 1975) or smooth muscle cells (Campbell, Uehara, Mark and Burnstock, 1971) are valuable indices of cell origin. Cell products such as intracellular secretory granules or collagen may give further information of cell type. Gershman and Rosen (1978) brought attention to the fact that cell-to-cell contacts may vary with time in culture and that cell position in the culture may alter the cell contacts made.

Monolayer cellular outgrowths were examined for specialised cell contacts known to be characteristic of epithelial cells, namely desmosomes with associated tono-filaments (Farquhar and Palade, 1963).

7.2.2 Cell Adhesion

Most mammalian cells are anchorage dependent, that is cell division only occurs if the cells are attached to a substrate (Pearlstein, 1978). The mechanism by which cells adhere to each other or to the matrix has long been a problem in cell biology (Weiss, 1969). The possible nature of adhesive factors (Weiss, 1971 ; Culp and Black, 1972), the role of micro-exudates (Maslow and Weiss, 1972 ; Yaoi and Kanasaki, 1972) the role of cyclic AMP (Grinell, Milan and Srere, 1973) as well as the effect of varying substrata (Harris, 1972 ; Harris, 1974) have all been regarded as significant.

Extracellular material both between cells and between cell and substratum may provide information concerning cell origin and give insight into the mechanism of cell attachment to the substratum.

Biochemically, proteins and proteoglycans may play a role in the adhesion process (Moscona, 1974). Recent work using baby hamster kidney (BHK) cells (Grinell, 1976) has suggested that a serum-derived protein itself becomes affixed to the substratum and plays an important role in cell attachment and spreading. With chinese hamster cells, adhesion to glass is a temperature dependent, energy requiring process involving uncharacterised proteins (Juliano and Gagalang, 1977).

The physical basis of cell adhesion to substratum has been investigated by the use of scanning electron microscopic techniques and carbon-platinum replica techniques (Revel, Hoch and Ho, 1974). Cell attachment points to substrata were found to be uniformly distributed along the underside of cells with, in BHK cells, heavy bundles of microfilaments parallel to the long axes of cells terminating in cell projections. Tryptic digestion left large portions of the cell underside remaining. These areas, known as substrate attached material (Culp and Buniel, 1976) indicate that the site of action of trypsin is on the cytoskeleton. Cytoskeletal filaments serve to anchor nuclei intracellularly (Lehto, Virtanen and Kurki, 1978).

Collagen has been investigated in murine cell lines since it was thought it may play a role in cell adhesion to substrate (Culp and Benusan, 1978). These studies failed to demonstrate sufficient collagen at adhesion sites to account for the cell-substrate adhesion process.

Stamatoglou (1977) using ruthenium red staining of a mouse tumour cultured on Melinex found a strand-like filamentous material between cell and substrate. Its character was undetermined but its position suggested a function in adhesion. Franks and Wilson (1970) and Franks and Cooper (1972) had previously described such an extracellular filamentous material in cultured cells.

Some workers have investigated cell adhesion from the viewpoint that the substratum may influence cell behaviour through a physical effect on the cell surface or via an influence on cell metabolism (Macieira-Coelho and Avrameas, 1972 ; Macieira-Coelho, Berumen and Avrameas, 1974). These authors concluded that substrata did not directly affect cell metabolism. Substratum surface charge probably influences cell behaviour as a consequence of its effects on cell adhesion.

The variables which can alter cell-to-cell and cell-to-substrata adhesion are poorly defined though recent advances have led to the importance of such macromolecules as fibronectin being recognised. Culture conditions were standardised in the present studies. Emphasis was placed on cell-to-cell contacts as an index of cell origin. The undersurface of cells was studied in less detail than cell-to-cell contacts which were studied by serial sectioning. An attempt was also made to study the contents of the extracellular space.

7.3 ULTRASTRUCTURAL ANALYSIS OF SALIVARY GLAND CELLS GROWN IN VITRO

Newborn mouse submandibular glands cultured under standard conditions of media and substrata were used throughout. All preparations used were embedded *in situ* without removal from the glass on which they have grown. This technique allows the

distribution of basal lamina-like material to be best seen (Franks, 1972). Following embedding (Luft, 1961) coverslips were removed by hydrofluoric acid dissolution. Blocks were sectioned and stained according to the method initially used for cells cultured on polycarbonate membranes (Nopanitaya, Charlton, Turchin and Grisham, 1977). Attempts were also made using the method of Sykes and Basrur (1971) to study the ultrastructure of cells cultured on Melinex. These authors had suggested that for some cells (rat embryo muscle) growth equivalent to that on glass could be achieved and ultrastructural studies could be performed without disrupting the monolayer. The present study, however, found no value in the extra steps involved, and thus concluded, as had Firket (1966), that Melinex is inefficient as a substratum.

The present study has combined investigations of cell structure, cell-to-cell contacts and the identity of extracellular molecules :

(a) Evidence was obtained for Type I and Type II epithelial cells originally described by Franks and Wilson (1970) for mouse salivary cells in vitro. Type I cells have rounded nuclei, short cell processes and relatively few lysosomes and mitochondria. Type II cells have a more convoluted nucleus with clumps of chromatin throughout the nuclear matrix, many lysosomes and dilated cisternae of rough endoplasmic reticulum. Cells with such features are shown in Figures 25 and 26.

(b) Cell-to-cell contacts, namely desmosomes, were investigated for their value as determinants of cell origin.

Desmosomes were recognised as regions of cell contact in which the cell membranes of adjacent cells contained a central electron dense lamina and intracellular tonofilaments (Fig. 27). Hemi-desmosomes were not observed. No regular quantification of the number of desmosomal contacts present was attempted but approximately 10% of cells examined had such specialised cell contacts. These contacts (Farquhar and Palade, 1963) indicate that the cells have an acinar, ductal or myoepithelial cell origin.

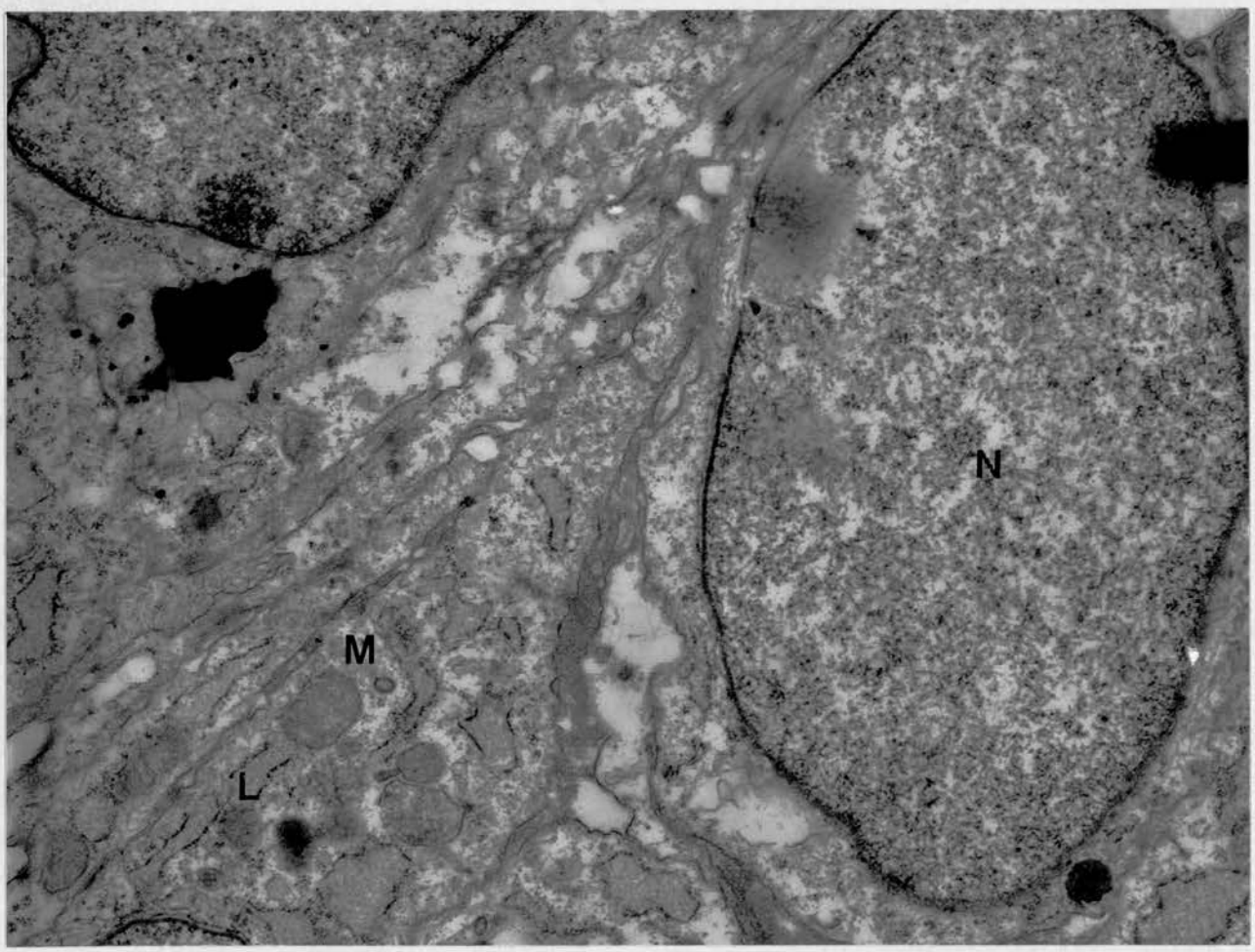


Figure 25

Ultrastructural appearance of mouse salivary gland *in vitro* showing rounded nuclei (N), short cell processes and relatively few lysosomes (L) and mitochondria (M). This appearance is consistent with Type I epithelial cell described by Frank and Wilson (1970).

X 16,000

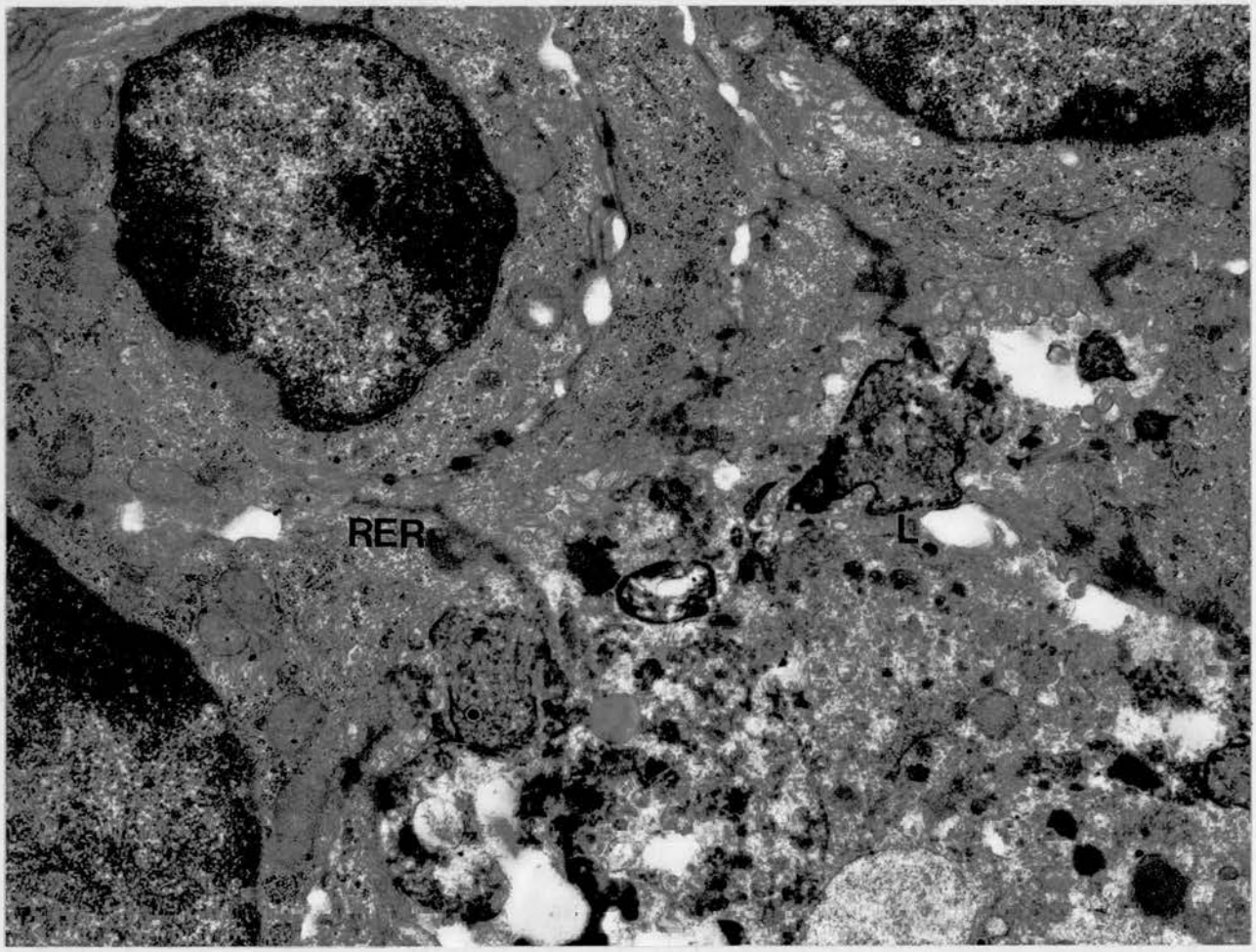


Figure 26

Ultrastructural appearance of mouse salivary cells *in vitro* showing a convoluted nucleus (N) with clumps of chromatin throughout the nuclear matrix, many lysosomes (L) and dilated cisternae of rough endoplasmic reticulum (RER). This appearance is consistent with the Type II epithelial cell described by Franks and Wilson (1970).

X 6,000

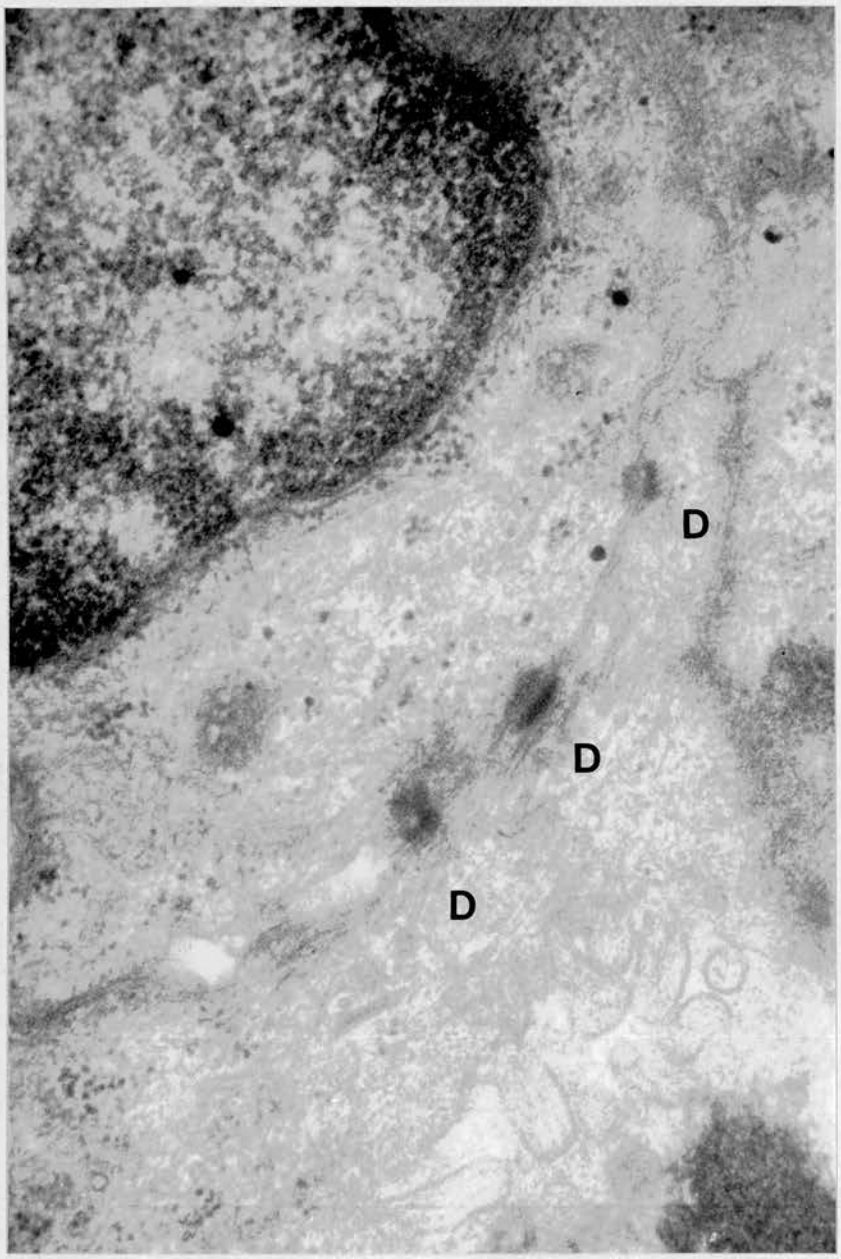


Figure 27

Desmosomal cell-to-cell junctions (D) present between mouse salivary cells *in vitro*. These junctions indicate a ductal, acinar or myoepithelial cell origin for these cells.

X 18,000

(c) Studies of extracellular molecules may provide extra evidence for cell origin. In Figure 28 a high power electron micrograph of extracellular material is shown. The periodic structure suggests that most of the extracellular material is collagen but less well defined areas may indicate that elastic tissue is also present. This elastic tissue appears homogeneous and shows no evidence of periodic structure (Toner and Carr, 1968). Numerous collagen elements are present between some cells and this abundance of material between cells presumably of connective tissue origin contrasts with epithelial tissues in which little extracellular material is found. Since fibroblasts are normally the source of collagen then the presence of cross-striated collagen fibres adjacent to cells is evidence for a connective tissue origin of the secreting cells. By definition (Federoff, 1967) a cell of appropriate morphology would then change from being considered fibroblast-like to be defined as a fibroblast. The undersurface of cells was investigated for specific cell attachment sites. An indistinct matrix was observed beneath some cells. Tangential collagen fibres were present between some cells and the substratum. This collagen exhibited the characteristic repeated banding pattern of 640Å which is believed to arise from the ordered parallel stacking of elongated tropocollagen molecules.

In conclusion, this aspect of the work has shown that fibroblasts and epithelial cells are present in the cellular outgrowth of newborn mouse submandibular salivary gland explants. The cells can be distinguished by functional and ultrastructural criteria.

7.4 ISOTOPIC, HISTOCHEMICAL AND IMMUNOLOGICAL APPROACHES TO THE IDENTIFICATION OF INDIVIDUAL CELLS

7.4.1 Autoradiographic Studies

Use of radio-isotopes offered yet additional means of distinguishing cells in the cellular outgrowth as well as providing further information on the dynamic aspects of that outgrowth. Three principal groups of studies were undertaken : identification of cells in DNA synthesis, preliminary studies

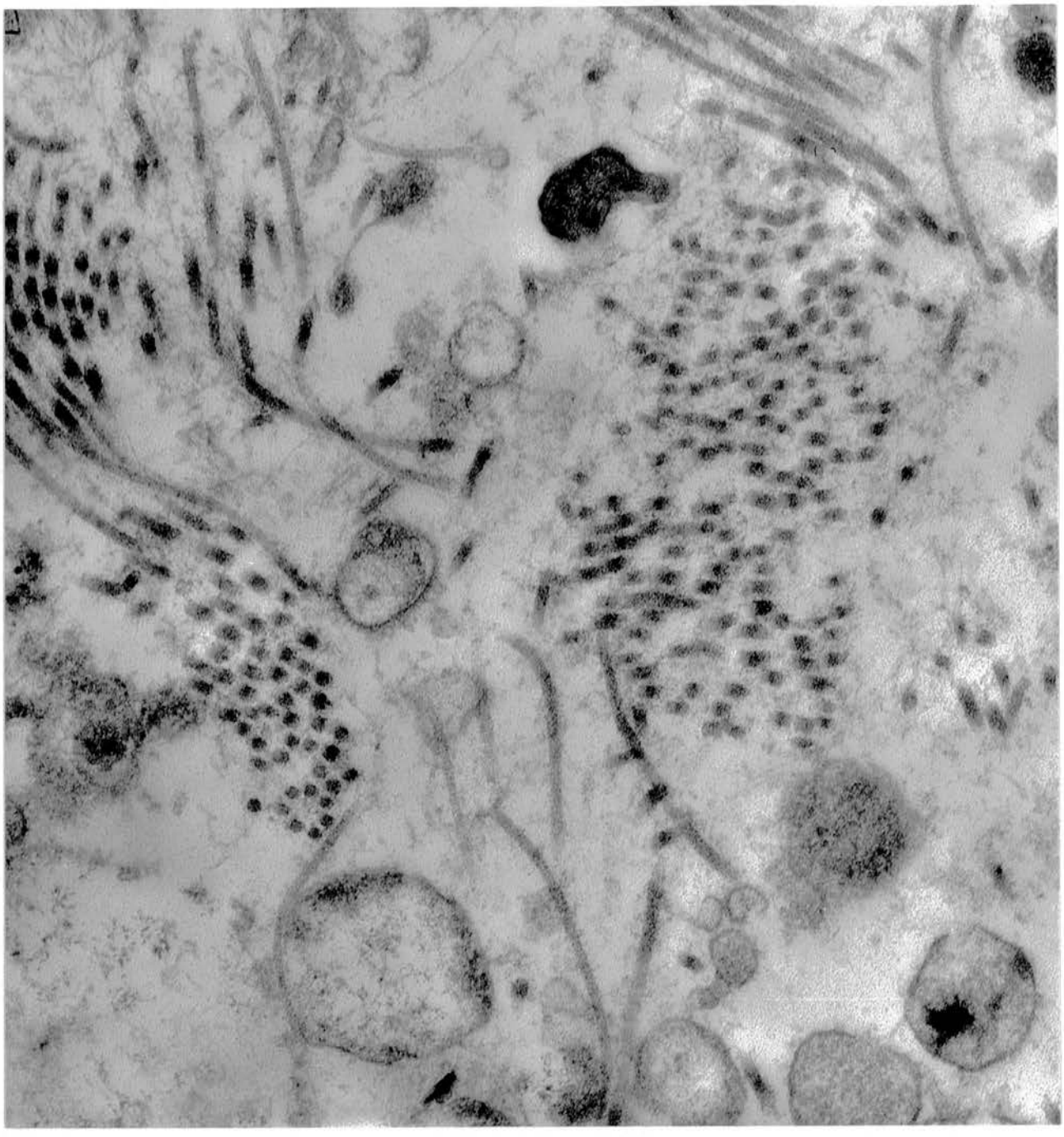


Figure 28

High power electromicrograph of the extracellular space between mouse salivary gland cells *in vitro*. A characteristic periodic structure is present in some of the material suggesting this is collagen whilst other areas show a more homogeneous appearance consistent with that of elastic tissue.

X 60,000

of protein synthesis and adrenergic receptor status of the cultured cells.

These studies used tritiated-thymidine (Radiochemical Centre, Amersham, England) at 1 $\mu Ci ml^{-1}$ and of specific activity 50 Ci ml^{-1} pulsed for 6 hour periods up to 24 hours on the 4 day old cultures of neonatal mouse submandibular gland. The technique employed is described elsewhere (Marshall, Lamey and Ferguson, 1980). Standard criteria for cell labelling were adopted (De Robertis et al. 1948). Secondly, by using tritiated proline and tryptophan the possibility was studied of further identifying cell types on the basis of protein synthesis. Collagen, the principal structural element of connective tissue is important in this study for two reasons : firstly, the extracellular insoluble form has a distinct fibrillar appearance and staining characteristic by light and electron microscopy. Collagen production may provide one method of cell attachment to the substratum. Secondly, the mechanism of collagen biosynthesis has been extensively studied in several fibroblast lines (Tsai and Green, 1972 ; Goldberg, Epstein and Sherr, 1972). The potential therefore was available for localising sites of proline uptake into cells and perhaps utilising this as an index of cell origin.

Tryptophan is an amino acid which in the mouse submandibular gland is localised by protein synthesis exclusively in the granular ductal cells (Doine and Fava-De-Moraes, 1979). In addition its presence is relatively unaffected by the hormonal status of the animal and would therefore be expected to persist in vitro. Granular cell uptake of labelled tryptophan may thus not only localise these cells in a mixed cell population but also permit subdivision of ductal cells on the basis of their rates of protein synthesis.

Thirdly, tritiated isoprenaline was included in this autoradiographic study to determine whether cell receptors bound the drug. Uptake in any residual sympathetic nerve endings would be expected since some authors consider that direct cell-to-neuronal contacts occur in vitro (Friedmann and Hodges, 1975).

Thus isoprenaline binding may be used to localise receptors on ductal epithelial and other cells. The findings in this study were that by using a 12 hours pulse of 1 μCi ml^{-1} ^{3}H-thymidine on day 4 of a neonatal mouse submandibular gland culture, there was more cell division at the periphery of the cellular outgrowth than in the region adjacent to the explant. (Table 6). In Figure 29, labelled cells at the periphery and around the explant are seen. Such autoradiographic studies provide evidence against the concept that cell flattening and migration were the means by which growth of an explant culture was achieved (Willmer, 1958). None of the other isotopically labelled molecules was of value in differentiating between cell populations.

7.4.2 Histochemical Enzyme Analysis

Table 7 outlines the results of the standard *in vitro* histochemical reactions of 7 day old outgrowths of neonatal mouse salivary gland explants and monolayers of NCTC 929, HeLa and human lamina propria established cell lines. Isocitrate dehydrogenase gave a reaction producing dense purple granules in the cytoplasm of some cells and appeared, as did the arylsulphatase reaction to give two cell populations ; one reacting strongly and the other weakly producing less than 5 reacting sites per cell. Arylsulphatase activity was predominantly located in the epithelioid cells. The reaction for 11 βHSD is shown in Figure 30.

7.4.3 Immunofluorescent Markers

In some diseases salivary epithelial cells have antibody directed against them. This observation offers a further means of making cellular distinctions. As outlined in 5.2.14 sera were obtained from patients with Sjogren's syndrome who were proven to be strongly positive for the presence of circulating salivary duct antibody.

Figure 31 illustrates the reaction obtained with this human salivary duct antibody directed at cultures of neonatal

TABLE 6

Differential incorporation of labelled thymidine in areas of explant cellular outgrowth

	Number of mitoses per 100 cells	Number of cells retaining tritiated thymidine per 100 cells
Periphery i.e. outwith one diameter of explant	3	13
Adjacent i.e. within one diameter of explant	1	6

Explant Periphery

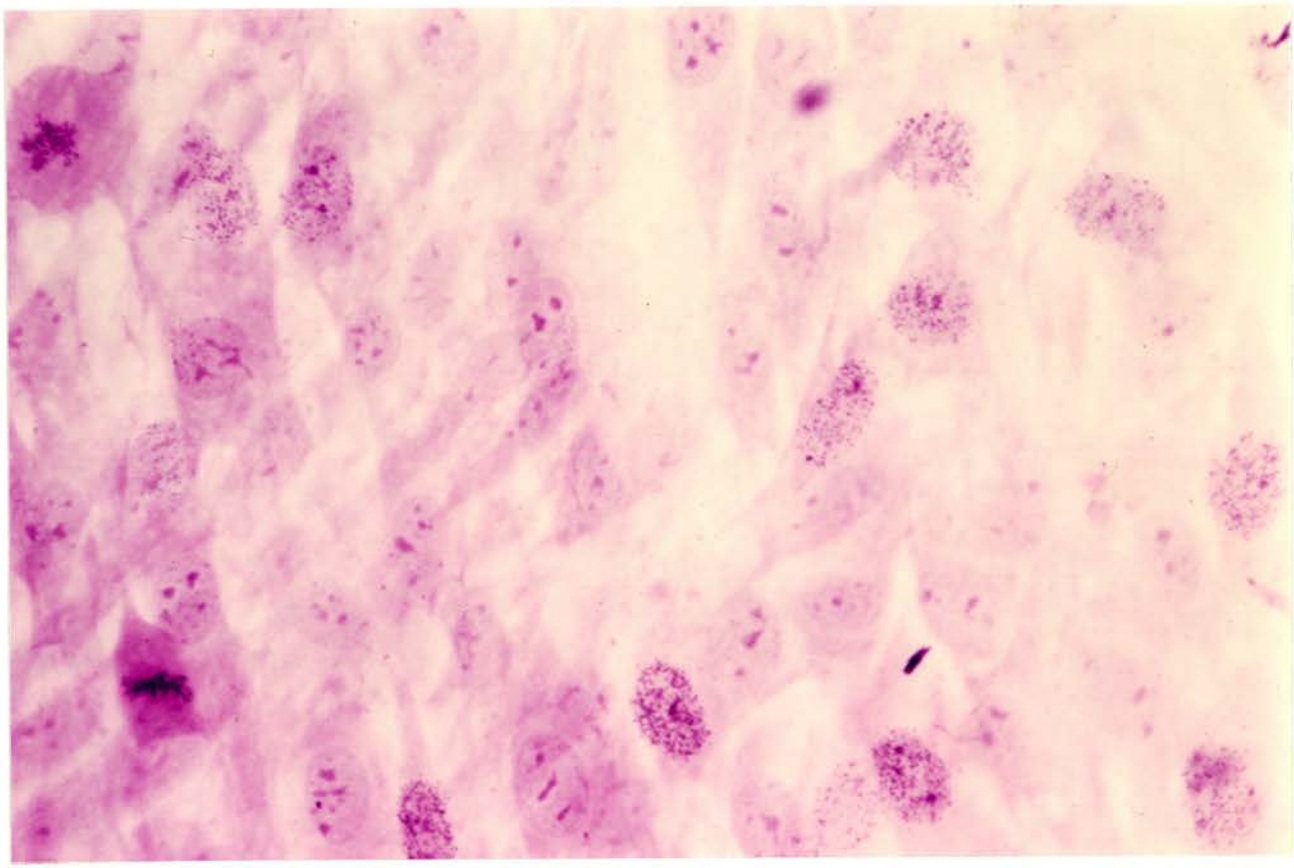


Figure 29

Autoradiograph obtained by pulsing cultured cells of mouse submandibular gland with tritiated thymidine. The cells which have retained tritiated thymidine are predominantly at the periphery suggesting that most cell division is occurring in this area.

X 460

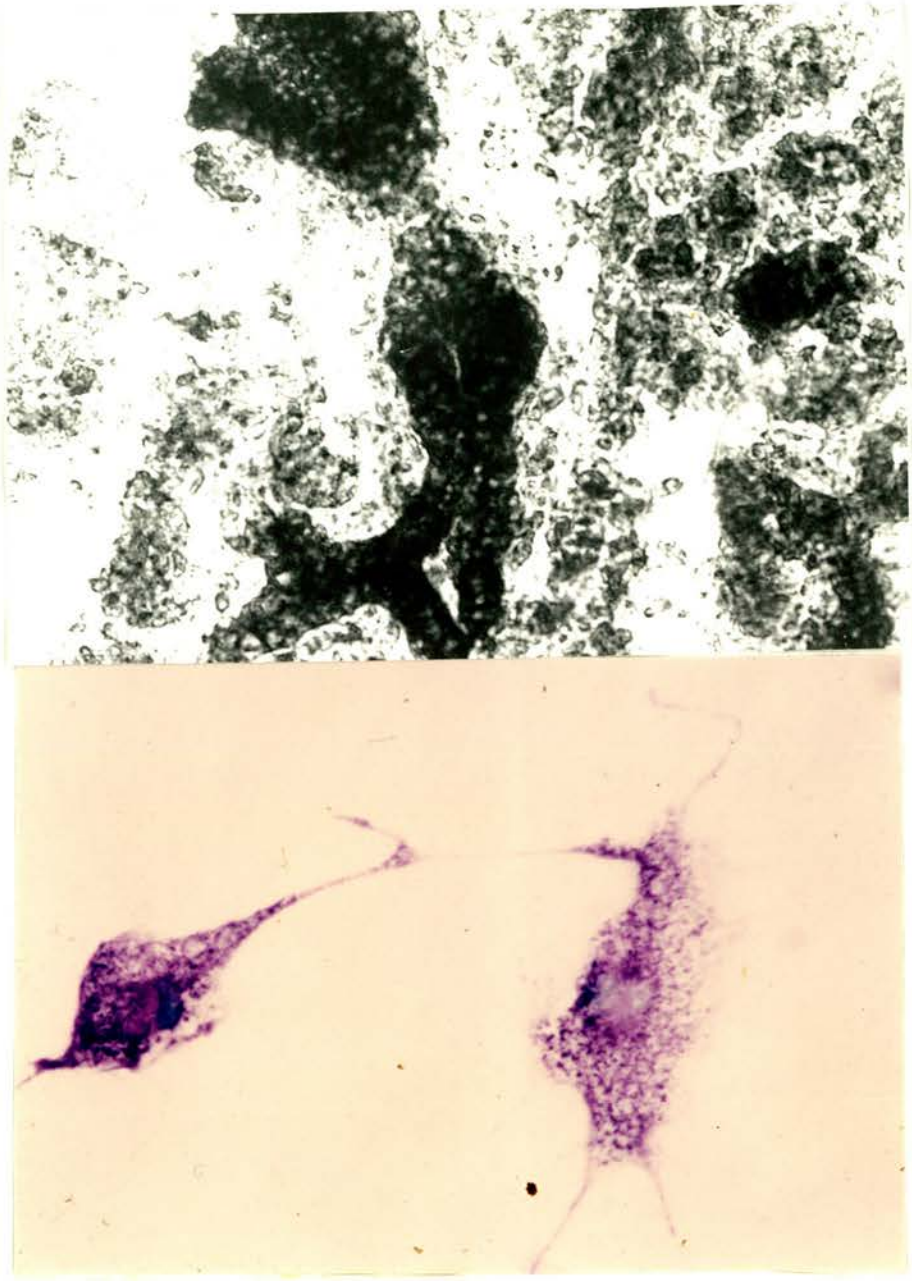
TABLE 7

Histochemical Results Showing Absence (-), Presence (+), or Equivocal (±)

Reaction for Each Enzyme Investigated

Culture	Succinate Dehydrogenase	Isocitrate Dehydrogenase	Glucose 6 Phosphate Dehydrogenase	αKeto Glutarate Dehydrogenase	11β Hydroxysteroid Dehydrogenase	Arylsulphatase (A + B)
Submandibular gland	+	+	+	-	+	+
Sublingual gland	+	-	+	+	-	-
Parotid gland	+	+	-	-	+	-
HeLa	+	-	+	±	-	-
NCTC 929	+	-	+	-	-	-
Lamina Propria	+	-	-	-	-	+

The methods are described in the text.



A

B

Figure 30

Histochemical reaction for 11 B HSD in frozen section (A) and in culture (B) of newborn mouse submandibular gland. A positive reaction is indicated by the formazan precipitation present in the cytoplasm. This reaction (B) indicates a ductal origin for these cells which as can be seen have a varied cell morphology.

A X400
B X500

Figure 31

Immunofluorescent reaction of mouse submandibular gland cultures to salivary duct antibody used in an indirect technique. This reaction indicates a ductal cell origin for these cells which again display a varied cell morphology indicating the need for specific criteria for identifying such cells.

X 300

mouse submandibular gland. The reaction in frozen sections is highly specific for the ductal component of the gland. In cultures, not all the cells giving a positive reaction are epithelioid. This indicates that ductal epithelial cells are present in the cellular outgrowth and *in vitro* can have an epithelioid or fibroblast-like morphology, stressing the need to search for unambiguous markers.

7.5 THE ROLE OF THE DIFFERENT CRITERIA IN IDENTIFYING SALIVARY CELL POPULATIONS

Gross cell morphology can be altered by changes in the cell environment (Folkman and Moscona, 1978). Even in established monoclonal cell cultures morphology is not constant. It is accepted that morphology alone is an unreliable criterion for cell identification. For example, by diameter ratios the epithelial cell line, HeLa, which was included for comparison, contained a proportion of fibroblast-like cells ; the converse was true for both the human lamina propria fibroblast line and the established NCTC 929 mouse fibroblast line. Cultures from all three mouse salivary glands contained both types of cells in varying proportions. Epithelioid cells were found scattered throughout the cellular outgrowth including the region immediately adjacent to the explant.

The presence of two apparent morphologically-distinct populations, as identified by the frequency distribution of cells with a given diameter, indicates that if more than two cell populations were present, they are morphologically indistinguishable from epithelioid or fibroblast-like cells by the present classification. There is evidence that the bimodal distribution does not reflect the distribution of epithelial and mesenchyme-derived cells in the outgrowth. This conclusion is substantiated when the relationship between cell morphology and a positive reaction to 11 βHSD or to salivary duct antibody is considered. It can clearly be seen for example in Figure 31 that some cells reacting with salivary duct antibody have an epithelioid character and others a fibroblast-like morphology.

The search for ultrastructural features characteristic of one cell type was undertaken to identify cells independently of gross morphology.

Desmosomes with tonofilaments are found in a proportion of cells in the cellular outgrowth from the salivary gland explants. This indicates epithelial cell origin (Farquhar & Palade, 1963). There are three practical disadvantages of using this as the only criteria for cell origin : it is both time-consuming and does not readily permit reconstruction of the overall position of any cell with its contacts in the cellular outgrowth. Thirdly, desmosomes in non-keratinised oral epithelium (Chen, 1970) can occupy less than 20 per cent of the plasma membrane. There is thus a probability of missing such attachments unless serial sections are examined.

The autoradiographic studies, although not providing evidence for cell origin in a mixed culture, have provided quantitative information concerning the means by which cellular outgrowth is achieved. The larger amount of uptake of tritiated thymidine at the periphery of the cellular outgrowth may be a reflection of loss of contact inhibition. The present work has shown that procedures which in vivo localise tryptophan to certain cells in the salivary gland (Sreebny and Meyer, 1964 ; Azuma, Sato and Maruyoma, 1980) are not as useful in vitro. Tryptophan uptake into granular tubules would be expected as would proline uptake into collagen made by fibroblasts. An antibody to prolyl hydroxylase, an enzyme involved in collagen synthesis, may have been useful but collagen synthesis is not confined to cells of mesenchymal origin (J. O'D. McGee. Personal Communication) and this approach was not pursued. The present studies show that ^{3}H thymidine will localise dividing cells, and give information on how the cellular outgrowth develops. The labelled amino acid and transmitter studies showed that the methods were too non-specific for cell identification.

Additional histochemical reactions may also be of value in identifying cell populations. The negative reaction for

isocitrate dehydrogenase in the fibroblast cell lines may be useful in conjunction with other tests. Fresh sections of mouse submandibular gland stained intensely for succinic dehydrogenase (Ferguson, 1966) and the reaction was predominantly localised to the intralobular ducts. In culture, an intense cell staining reaction was also found for this enzyme in some cells. Duwey (1958) considered the distribution of succinic dehydrogenase to accord well with that of mitochondria ; in rat parotid the duct cells demonstrated marked activity whilst the acinar cells reacted only moderately.

Arylsulphatase, whilst not giving a reaction specific for one cell type gave a heavy and light response indicating two cell populations. None of the other enzymes investigated was of obvious value in cell type determination.

A conclusion from the histochemistry study is that the epithelial component in the cellular outgrowth from primary explants of neonatal mouse submandibular and parotid gland is ductal in origin. This contrasts with previous work which believed them to be acinar

CHAPTER 8

CELLULAR ANALYSIS OF THE EXPLANT OUTGROWTH IN THE PRESENCE OF NEUROTRANSMITTERS

8.1 AUTONOMIC INNERVATION AND SALIVARY GLAND FUNCTION AND GROWTH

Evidence for salivary gland function being under the control of the autonomic nervous system comes from several workers. Salivary gland growth has been altered both by surgical denervation and exogenous administration of sympathomimetic amines. Thus Snell (1960) demonstrated submandibular salivary gland atrophy in the rat following parasympathectomy, whereas Wells, Handleman and Milgram (1961) showed inhibition of the normal growth of the ipsilateral submandibular gland following unilateral sympathectomy. Selye, Veilleux and Cantin (1961) and Pohto (1964) demonstrated that exogenously administered isoprenaline induced salivary gland enlargement in the rat. Brenner and Stanton (1970) considered this salivary gland enlargement to be mediated via beta adrenergic receptors. All these were *in vivo* experiments from which it is difficult to conclude directly that any drug administered is having a primary effect on the salivary glands.

Few publications are available describing the effect of exogenous compounds on salivary gland growth *in vitro*. Malamud and Baserga (1967) unsuccessfully attempted an organ culture system for rat and mouse submandibular salivary glands. Kreider (1970) used a tissue culture system involving monolayer cultures of rat submandibular and parotid salivary glands and demonstrated a moderate stimulation of ^{3}H-thymidine uptake into cells following exposure to isoprenaline. David and Maury (1978) investigated the response of ion fluxes to noradrenaline, adrenaline, isoprenaline and 5-HT (serotonin) in dispersed rat parotid cells, but were not concerned with alterations in salivary gland growth produced by these agents.

Hitherto, the lack of a reliable culture system probably accounts for the few attempts to correlate quantitatively, the effect of agents such as isoprenaline on a tissue culture system in which both the range of growth and cell populations present are known and identifiable. Kreider (1970) did not specifically identify the cell population present in the rat salivary monolayer cultures employed. On the basis of morphology, which has already been discussed here as an unreliable criterion for identifying cultured salivary epithelial cells, Kreider considered the rounded cells to be acinar cells which had lost specialised functions such as amylase production. This raises the question of whether these cells dedifferentiate during culture periods of several cell divisions. In 36 hour cultures Kanamura and Barka (1975) used ultrastructural features and endogenous peroxidase activity to identify acinar cells confirming that for this limited period at least, specialised cell function can be maintained. In the present study ultrastructural, histochemical and immunological criteria have been used to identify ductal cell populations present in the cellular outgrowth. The response of these cells to dopa, noradrenaline, isoprenaline, acetylcholine, carbachol and 5-HT (serotonin) was assessed stereologically. The total growth area was measured planimetrically and was related to changes in cell number.

8.2 Measurement of Growth

The method for culture of newborn mouse submandibular salivary gland cells has been described in Chapter 6. In these following experiments growth was measured on day 7 by the planimetric method. Column analysis was performed to ensure that a change in area reflected a change in cell number and not in spread area per cell (Greenberg, Grove and Cristofalo, 1977). The phenomenon of cell spreading is a known effect of some surface active drugs.

8.2.1 Selection of neurotransmitters and related agents

At the outset of the study the weight of evidence favoured the presence of adrenergic and other receptors on salivary cells. In addition to isoprenaline which is known to produce salivary gland enlargement in mice *in vivo* (Brown-Grant, 1961) agents were chosen according to their mode of action. Noradrenaline has a direct alpha adrenergic receptor effect whilst dopa, a precursor of sympathomimetic amines, acts indirectly. Acetylcholine and carbachol have para-sympathomimetic activity but the latter is much less susceptible to enzymatic inactivation and thus would be expected to have a longer duration of action. Serotonin (5HT) acts via different receptors than the previous agents. Any response to 5HT would be expected not to involve sympathetic or parasympathetic cell receptors.

8.2.2 Incubation Regime

In these experiments, physiologically-saturating amounts of dopa, noradrenaline, isoprenaline, acetycholine, carbachol and 5HT were added sterile to the growth medium at the beginning of incubation to give a final concentration of 10^{-4}M. Prior to addition of growth medium containing any agent, the medium was agitated for thirty minutes to ensure even dissolution of agent. Agents, in this same concentration were also added on day 3 when the growth medium has changed and was thus present until cultures were analysed on day 7. Isoprenaline was added to other cultures in the same regime to produce a graded series of concentrations of 10^{-3}M, 10^{-4}M, 10^{-6}M, 10^{-8}M and 10^{-10}M. For each drug, control cultures received growth media alone.

8.2.3 Analysis of growth and epithelial cell populations

These studies sought to quantify the relationship between functional and morphological criteria of cell origin in the drug treated cultures.

Morphological studies were paired, each moiety receiving 11β-hydroxysteroid dehydrogenase histochemical stain (11βHSD) or fluorescent salivary ductal cell antibody (SDA) routinely to identify ductal epithelial cell populations. Results were expressed as the percentage of morphological epithelioid cells or enzyme - or antibody-reacting cells in a cell column. Diameters of individual epithelioid or fibroblast-like cells were measured to determine the relationship between cell size and growth in the presence of the neurotransmitters.

8.3 RESULTS

The growth response of cultured salivary gland cells to dopa, noradrenaline, isoprenaline, acetycholine, carbachol and 5-HT at concentrations of 10^{-4}M are summarised in Table 8 and illustrated in Figure 32. At seven days, growth in the presence of all the agents, except dopa and carbachol, was statistically less than that of control cultures. None of the agents produced an increase in growth.

The response to isoprenaline between 10^{-3}M and 10^{-10}M, is summarised in Table 9 and illustrated in Figure 33. At 10^{-3}M isoprenaline did not permit cellular outgrowth to occur but instead the explant appeared to subside, resulting in a flattened appearance resembling gland sections (organotypic character). A concentration of 10^{-4}M and 10^{-6}M isoprenaline diminished the growth rate but allowed outgrowth. The growth rate equals that of the controls at a 10^{-8}M and 10^{-10}M. Thus inhibition of salivary gland cellular outgrowth by isoprenaline is dose dependent, the inhibition diminishing around 10^{-6}M.

8.4 CHANGES IN EPITHELIAL CELL POPULATIONS IN RESPONSE TO SELECTED NEUROTRANSMITTERS

In the control cultures, ultrastructurally, the presence of desmosomes could be detected in about 10 per cent of cells in culture. The same frequency was observed in all the drug-treated cultures. This indicates that the neurotransmitters do not alter the apparent origins of cells in the cellular

TABLE 8

Inhibition by Neurotransmitters
of Mouse Salivary Gland Cell Outgrowth

Agents (10^{-4}M)	Monolayer Area (units)^2	Significance
Control	321	
Dopa	174.4	NS
Noradrenaline	151.4	$p<0.01$
Isoprenaline	86.4	$p<0.001$
Acetylcholine	176.8	$p<0.01$
Carbachol	196.4	NS
5-HT	139.6	$p<0.001$

NS = Not significant

Growth was in the presence of each transmitter applied for seven days.

Tissue area units : 550 units^2 = 4mm^2.

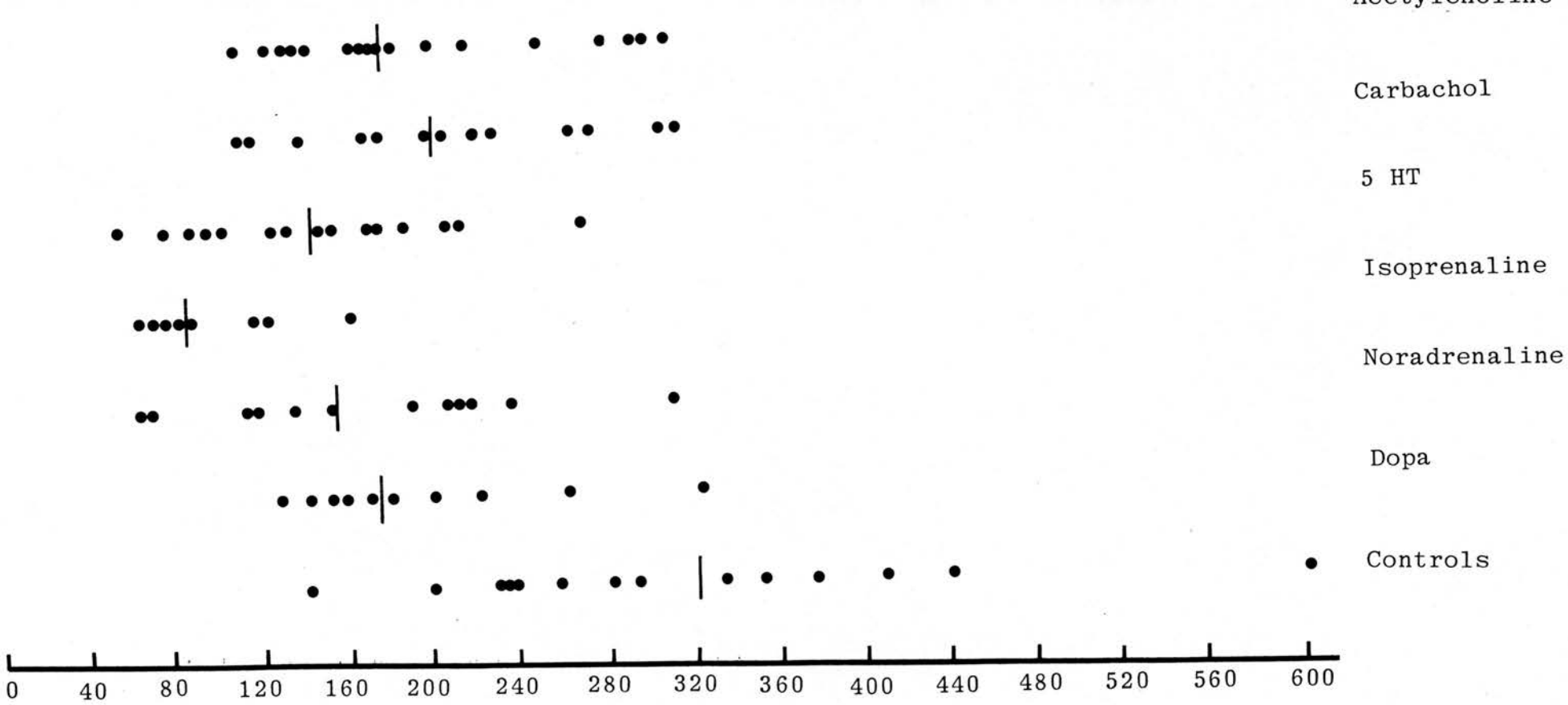


Figure 32

Scattergram illustrating the range of growth of newborn mouse submandibular gland cultured in the presence of selected neurotransmitters and related agents. The growth dimensions are those of the area of cellular outgrowth from the explant.

TABLE 9

Salivary Gland Explant Growth response in the presence of isoprenaline

Concentration (M)	Monolayer Area $(units)^2$	Significance
10^{-3}	No growth	$p<0.001$
10^{-4}	86.4	$p<0.001$
10^{-6}	183.4	$p<.05$
10^{-8}	197.8	NS
10^{-10}	188.4	NS
Control	320.0	

NS = Not significant

Conditions as described in text.

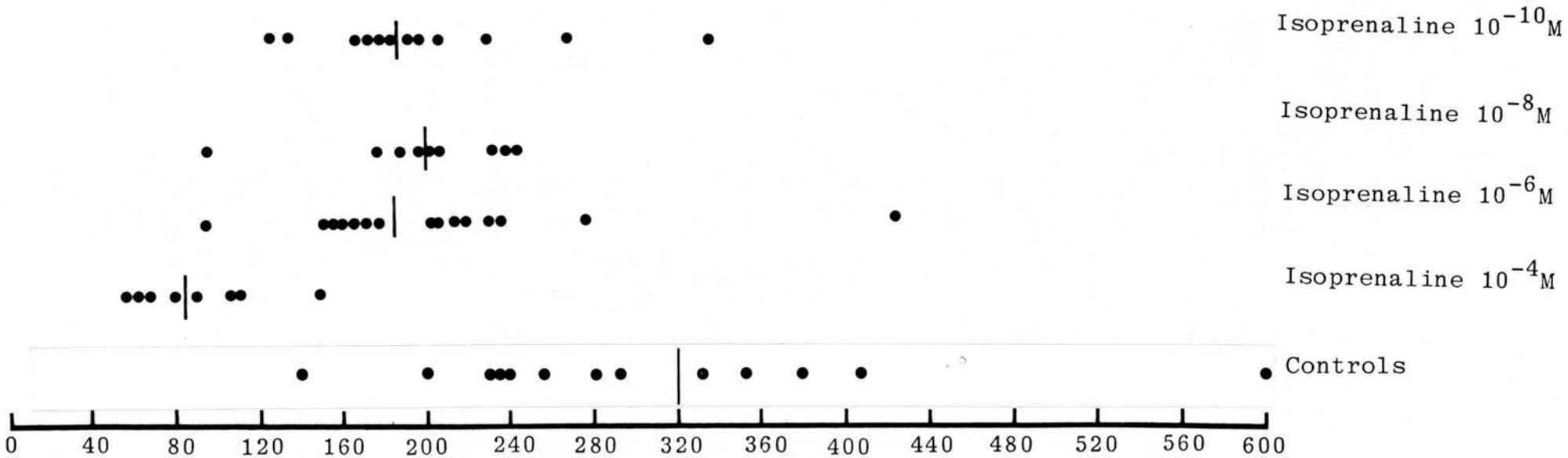


Figure 33

Scattergram illustrating the growth response of newborn mouse submandibular gland in the presence of a graded dose of isoprenaline.

outgrowth nor change the amount of desmosomal membrane present. Thus response to neurotransmitters as assessed ultrastructurally did not offer a means of amplifying any useful differentiation steps. The relationship between cells described as epithelioid and either the presence of 11βHSD or a positive reaction with salivary duct antibody has been shown in Chapter 7. Using these techniques on cells grown in the presence of neurotransmitters (Tables 10 and 11) there was observed an alteration in the number of ductal epithelial cells present. This could not have been deduced on morphological grounds alone and demonstrates that in vitro ductal epithelial cells may have an epithelioid or fibroblast-like morphology. Both techniques were used to verify changes in cell populations as detected by the ratio of epithelioid (E) to fibroblast-like (F) cells and were found to correlate well (Tables 10, 11).

Table 12 demonstrates that cell diameter in the epithelioid (E) or fibroblast-like (F) cell groups is not significantly changed by any of the agents used. The difference between the mean E and F cell diameters is significant ($p<.001$) throughout. Other data not shown here but resembling that shown in Chapter 7 (Figure 24) demonstrated a bimodal distribution of cells in control and treated cultures which did not show kurtosis.

Neurotransmitters at 10^{-4}M altered the ratio of epithelioid to fibroblast-like cells (E/F), summarised in Table 13. All agents, except dopa and isoprenaline, altered the E/F ratio significantly from that of the control cultures. Table 8 shows that isoprenaline suppresses growth but it was found not to alter the E/F ratio throughout the dose range 10^{-4}M to 10^{-10}M.

Cell viability as assessed from routine trypan blue exclusion tests at day 7 was consistently around 85 per cent for both control and drug treated cultures.

TABLE 10

Typical relationship between epithelioid (E) or fibroblast-like mouse salivary gland cell morphology (F) and the intracellular presence of 11βhydroxysteroid dehydrogenase (11βHSD)

Culture	Percentage of Epithelioid cells in a radial column	Percentage of E or F cells positive for 11β HSD in a radial column	
		E*	F*
Control	30	22	2
Carbachol 10^{-4}M	14	12	2
Isoprenaline 10^{-4}M	35	19	4

Cultures were examined at 7 days.

* Percentage values refer to E or F populations as a percentage of the total cells in a column (Chapter 5). The values are average values derived from 10 cultures.

TABLE 11

Typical relationship between epithelioid (E) or fibroblast-like morphology (F) and positive reaction with salivary duct antibody (SDA)

Culture		Percentage of Epithelioid cells in a radial column	Percentage of cells positive for SDA in a radial column	
			E	F
Control		30	26	2
Carbachol	10^{-4}M	16	10	1
Isoprenaline	10^{-4}M	33	29	0

Conditions and methods are described in legend to Table 10 and Chapter 5.

TABLE 12

Typical relationship between mouse salivary cell diameters of epithelioid (E) and fibroblast-like (F) cells in control and treated cultures

	E Diameter			F Diameter		
Supplement	n	Mean	S.D.	n	Mean	S.D.
Control (nil)	87	10.4	2.0	190	21.5	3.5
Dopa	233	11.1	2.6	355	21.4	3.8
Noradrenaline	282	10.2	2.4	91	23.2	3.7
Isoprenaline	274	10.5	8.2	550	20.1	17.7
Acetycholine	256	10.9	2.1	298	21.3	4.3
Carbachol	53	11.1	2.2	340	21.2	13.6
5-HT	130	9.9	1.9	138	27.3	2.9

n = cell number

S.D. = standard deviation

E/F p = <.001

For convenience, diameters are given as the raw data obtained from the teaching head of the Ortholux microscope. With the sections used here 30 units diameter = 59μ.

TABLE 13

The percentage of epithelioid or fibroblast-like cells growing from mouse salivary gland explants in the presence of neurotransmitters

Agent, 10^{-4}M	Percent epithelioid cells in population	Significance
Control	31	
Dopa	39	NS
Noradrenaline	75	$p<0.001$
Isoprenaline	33	NS
Acetycholine	46	$p<0.001$
Carbachol	13	$p<0.001$
5-HT	50	$p<0.001$

NS = Not significant

8.5 DISCUSSION

At a concentration of 10^{-4}M none of the agents investigated increased growth from the salivary gland explant and all but dopa and carbachol suppressed growth below that of control cultures. There was no evidence of toxicity at 7 days in 10^{-4}M agents, as evidenced by inability to exclude trypan blue. This dose is considered non-toxic both to mouse salivary glands (Kanamura and Barka, 1975) and frog skin *in vitro* (Benson and Hadley, 1969). The inhibition of growth could be due to selection of cells surviving this concentration and which divide at a reduced rate. Alternatively, all cells could be affected and cell cycle time increased in a manner which, for isoprenaline at least, is dose-dependent.

Salivary gland development can proceed morphologically normally *in vitro* in the absence of neural innervation (Borghese, 1950 a ; Coughlin, 1975 a & b) so it is conceivable that neurotransmitters are more concerned with attainment of specialised secretory properties than morphogenesis, as has been suggested by Schneyer and Schneyer (1967). The evidence in the present study indicates that $>10^{-6}$M neurotransmitters exert inhibitory control over salivary gland growth *in vitro*. If this effect is receptor-mediated it is not limited to the adrenergic or cholinergic system.

Receptor antagonists were not studied, making difficult a comparison of the *in vivo* and *in vitro* growth response to such agents. Chronic administration of isoprenaline produces pronounced hyperplastic and hypertrophic enlargement of the salivary glands of the rat and mouse (Brown-Grant, 1961 ; Selye, Veilleux and Cantin, 1961) and asymptomatic enlargement of the parotid in patients receiving isoproterenol hydrochloride in the treatment of chronic bronchial asthma (Borsanyi and Blanchard, 1961).

There are probably two reasons why isoprenaline at doses expected to be pharmacologically active did not increase growth of murine salivary cells *in vitro* here. Firstly, in species other than mice isoprenaline is a less effective stimulant to growth of the submandibular than parotid gland (Byrt and

Glanvill, 1967). If true for mice, salivary gland explants in vitro this would be important. Secondly, the processes of hypertrophy and hyperplasia observed following isoprenaline administration in vivo is initially of the acinar cells (Schneyer and Shackleford, 1967 ; Barka, 1965) although ductal cell DNA synthesis occurs later. The number of acinar cells growing in the cellular outgrowth is unknown. Rat parotid studies (Burke and Barka, 1978) have shown no change in beta receptor number following stimulation with isoprenaline. Receptor specificity exists since isoprenaline isomers differ in their effects on DNA synthesis (Kirby, Swern and Baserga, 1969). In mice acinar cell damage has been considered part of the response to isoprenaline (Novi and Baserga, 1971 ; Simson, 1972) and may further deplete any acinar cells present in our cultures. It appears that isoprenaline in vitro is unable to increase salivary gland growth. The sialadenotrophic response observed in vivo may be the result of additional hormonal effects for with isografts of submandibular gland testosterone and isoprenaline together produced an increase in granular tubule cells whilst isoprenaline alone resulted in an acinar cell response (Hoshino and Lin, 1970). Further experiments will determine the relationship between the effects of isoprenaline and sex hormones in vitro. There is no distinguishing relationship between inhibition of growth and receptor type with agents other than isoprenaline. Acetycholine and noradrenaline produce similar inhibition of growth and differentially alter the E/F cell ratio. However, dopa and carbachol produce similar inhibition of growth but carbachol alters the E/F ratio in favour of fibroblast-like cells. Table 13 also shows that serotonin (5-HT) produces effects on the E/F ratio intermediate between noradrenaline and acetycholine and inhibition of growth intermediate between isoprenaline and carbachol. Whether these observations are manifestations of a specific selection of one or other cell type by differentially altering growth rates or by a process of E to F transformation is not clear. The use of histochemical indices to identify ductal epithelial cells suggests a true change in ductal epithelial cell numbers is being observed.

CHAPTER 9

HUMAN LABIAL SALIVARY GLAND GROWTH IN VITRO : MARKERS FOR DUCTAL EPITHELIAL CELLS AND THE QUANTIFICATION OF CELL POPULATIONS

9.1 INTRODUCTION

Transition from murine to human cultures

The murine studies described in Chapter 5 onwards have shown that murine salivary cells can be grown *in vitro*, and that cell populations can be identified by a combination of immunological, histochemical and ultrastructural criteria. The distinctions have allowed the unambiguous recognition of ductal cells in a mixed culture. The procedure of cell identification is complex but can be applied to normal and experimentally-supplemented cultures.

Once ductal cells had been identified, a problem was to decide whether similar procedures could be used to identify human salivary gland cell populations.

A study was needed to test this possibility. At the same time it would provide a means for deciding whether the murine culture system should be continued alone or developed in parallel with a human study.

Therefore, since human labial salivary glands offer an accessible source of gland tissue, an *in vitro* study was planned using biopsy specimens. An advantage of this study is that human labial glands have been recognised (Chisholm and Mason, 1968) as reflecting major salivary gland involvement in disease processes. Identification of cells had necessarily to be based on the current murine observations. In mice ductal epithelium converts cortisol to cortisone by the enzyme 11β-hydroxysteroid dehydrogenase (11β HSD) (Ferguson, Glen and Mason, 1970). This enzyme may therefore be an important marker of ductal epithelium in man.

9.2 DERIVATION OF HUMAN SALIVARY EPITHELIOID CELL LINES

Derivation of cell lines from the initial primary labial explant biopsy (Figure 34) was investigated.

The method for processing explants was similar to that used in the murine studies. Up to the third weekly passage, cells were plated on to cover-glasses but for subsequent passages the suspended cells were allowed to form monolayers on 25 cm^2 plastic flasks (Nunclon Nunc, Denmark). Epithelioid cells from the primary explant were derived by repeated tryptic digestion and mechanical dislodgement of fibroblast-like cells. At the end of the first week cultures were divided to give 3-4 separate lines. By the third subculture on glass a morphologically homogeneous epithelioid culture was obtained. After this, each line thus derived was maintained by weekly replating in standard medium and usually kept until around the 18th passage. If passaged on plastic the culture remains predominantly epithelioid. However, as with murine cells, the dehydration and fixing steps of the enzyme assays require the cells to be cultured on glass where epithelioid morphology was only maintained for about 3 passages. For this reason third glass passage cultures were used for the histochemical assays.

9.3 COMPARISON OF THE IN VITRO CELLULAR OUTGROWTH FROM HUMAN AND MURINE SALIVARY GLAND EXPLANTS

Medium 199 supplemented with 20 per cent newborn calf serum yielded greater growth of both human and murine cells, than with either 10 per cent or 20 per cent foetal calf serum supplements, or 20 per cent autologous serum.

Under the same medium conditions, the area of outgrowth at 7 days differed between mice and human explants. This difference was not diminished by changing the serum supplement. If the human cultures were left for ten days a comparable growth area was achieved. This phenomenon was not studied in

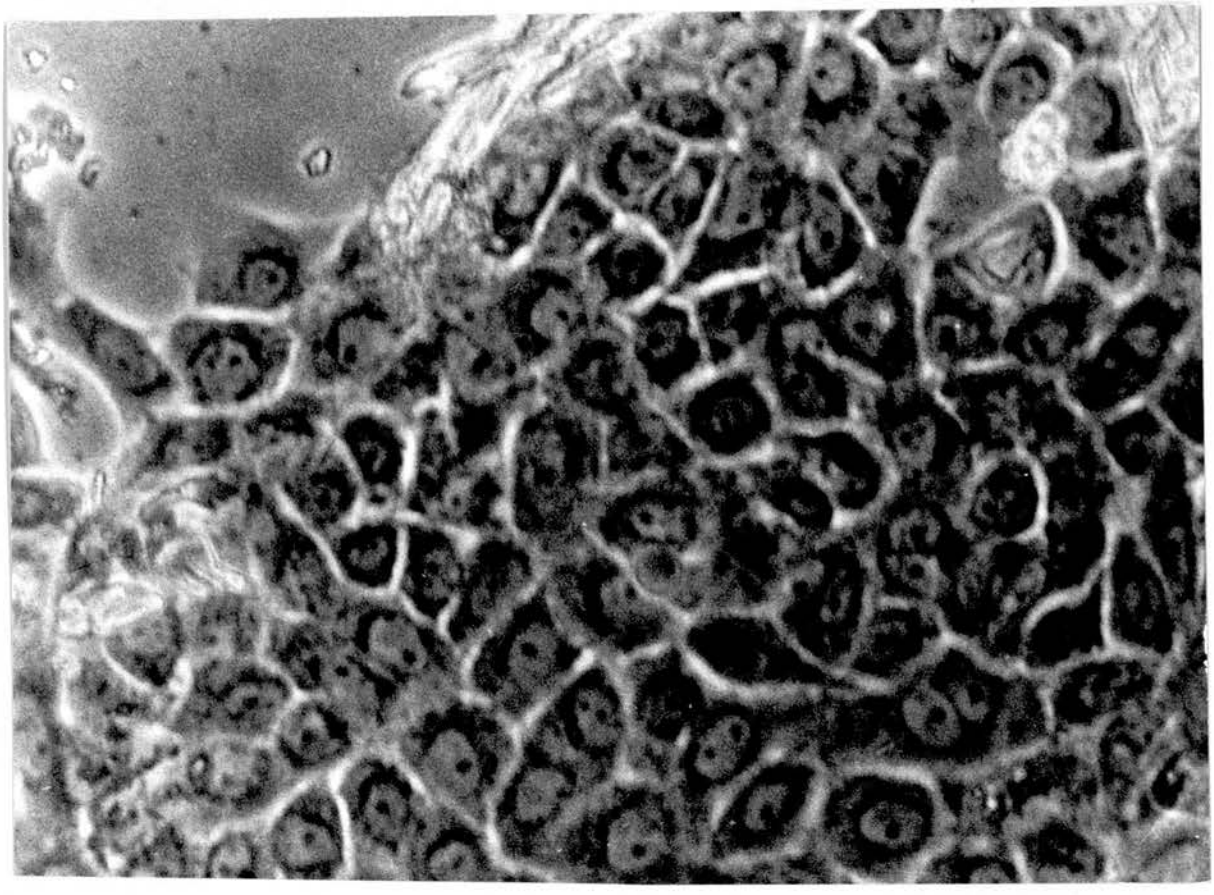


Figure 34

Monolayer cellular outgrowth produced from explant culture of human labial salivary gland. The cells have predominantly epithelioid appearance.

X 400

great statistical detail for the reason that the human cultures grew characteristically in a non-confluent manner, yielding monolayer clusters connected by cell strands. This is seen in Figure 35.

The differences between the culture characteristics of the human and murine cellular outgrowths are shown in Table 14.

An unexplained feature of the human cultures is the eccentric nature of the outgrowth. This could not be traced to abnormalities of the growth environment. Such factors as incomplete cleansing of coverglasses for culture, temperature gradients in the incubator, and supplemented serum concentration, were examined systematically.

The cell streams contained cells which had a fibroblast-like appearance together with epithelioid cells. By electron microscopy, collagen fibrils were seen but it is difficult to say precisely which cells in the monolayer these fibrils were related to. A new approach to this problem would be to use a monoclonal antibody cytotoxic for human fibroblasts in the presence of rabbit complement (Edwards, Easty and Foster, 1980) for cloning these cultures. A combination of this approach and enzyme histochemistry may allow the necessary distinctions to be made on several questions such as whether the observed pattern is a manifestation of epithelial-fibroblast interactions, and the role of differential cell growth rates within complex cell territories. As with murine cultures, the morphological appearances can be deceptive. For example, Figure 35 shows a fibroblast-like cell positive for 11β HSD indicating ductal epithelial cell origin. It was found from the lamina propria cultures, outlined in Chapter 7 , that human fibroblasts *in vitro* do not have this enzyme.

There is some evidence for differential cell growth rates. Figure 35 shows that predominantly fibroblast-like cells in these human cultures have labelled nuclei.

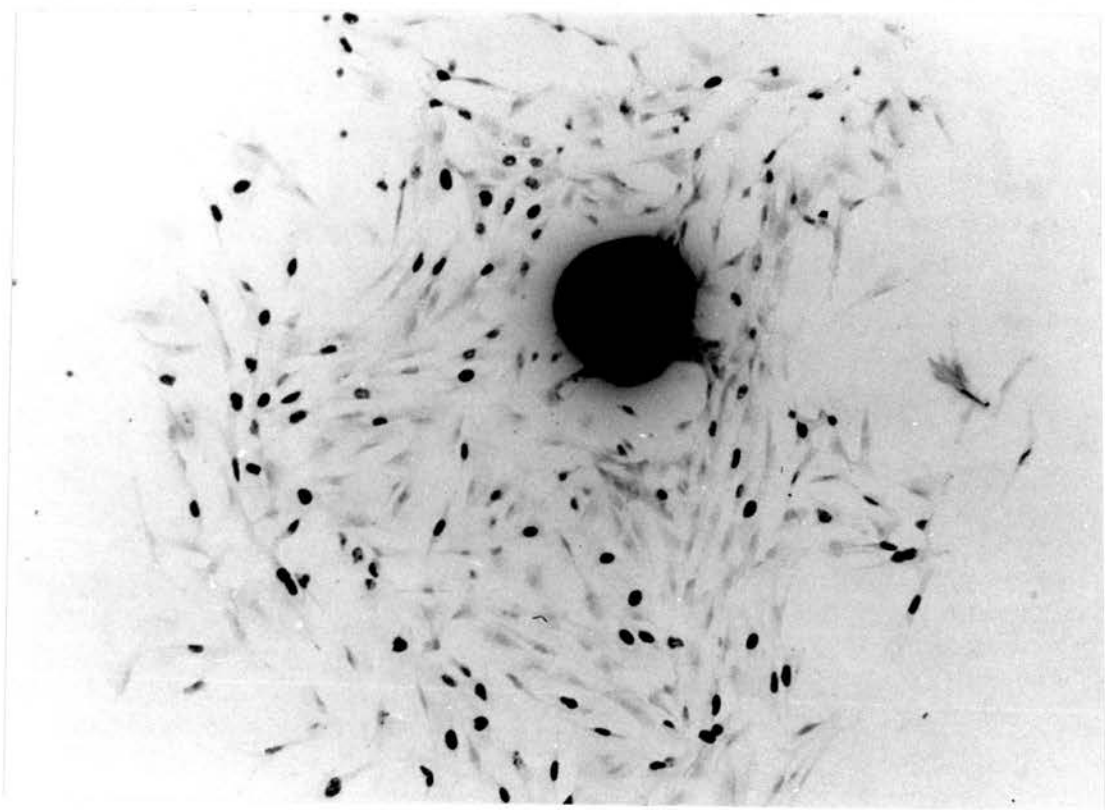


Figure 35

Autoradiograph obtained following pulsing of a human labial salivary gland culture with tritiated thymidine. The inability of the cells to utilise all the growth surface available is illustrated as in the diffuse distribution of cells retaining tritiated thymidine.

X 60

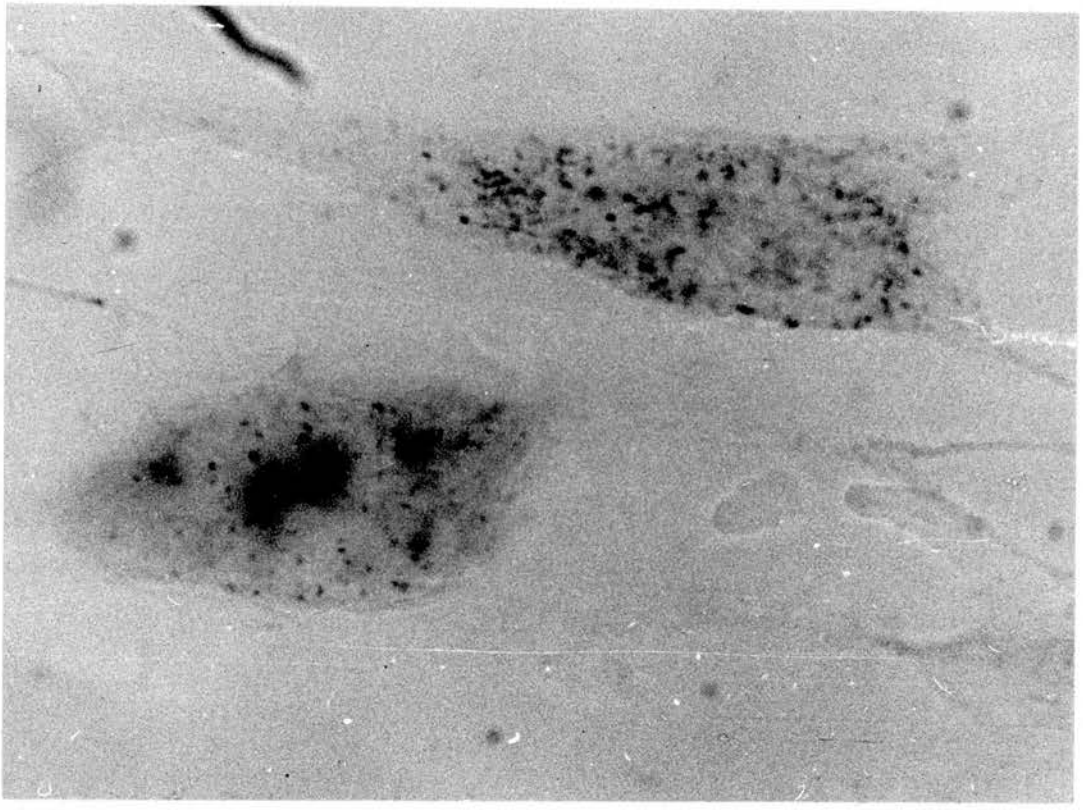


Figure 36

Histochemical reaction obtained to 11β HSD of human labial salivary cell cultures *in vitro*. A positive reaction is indicated by the intracellular deposition of formazan salt. The varied cell morphology of positive reacting cells emphasises again the need for absolute indices of cell origin.

X 600

TABLE 14

Comparative Culture Characteristics of Human Labial Salivary Gland In Vitro and Adult Mouse Submandibular Salivary Gland

		Human	Mouse
1.	Explant size producing cellular outgrowth	up to 1.0mm	less than 0.4mm
2.	Adhesion success rate	70%	80%
3.	Time of initial outgrowth	3 - 4 days	2 - 3 days
4.	Type of outgrowth	Eccentric	Concentric
5.	Manner of outgrowth	Spaced	Continuous
6.	Area 7 days	210 units*	330 units*
7.	Presence of sub-populations of cells	Yes	Yes
8.	Cloning ability	Yes	Yes
9.	Type of cell line derived	Epithelioid	Variable
10.	Response to ^{3}H	Periphery+	Periphery++
11.	Response to autologous sera	Variable	Less than bovine
12.	Response to foetal calf serum	Reduced growth	No growth
13.	Presence of desmosomes	Yes	Yes
14.	Presence of 11β hydroxysteroid dehydrogenase		
	a. primary explant	Yes	Yes
	b. clone	Yes	Yes
15.	Ability to convert cortisol to cortisone		
	a. primary explant	Yes	Yes
	b. clone	Not tested	Yes

* 4 mm^2 = 550 units

These arguments suggest that a concentric cellular outgrowth from murine explants could mean that a homogeneous cell population is growing from the explant. This possibility is not supported by the histochemical and immunofluorescence evidence cited in Chapter 7. At least two cell populations were identified in these concentric outgrowths : one ductal epithelial in origin, and the other by ultrastructural criteria contained a proportion of fibroblasts.

9.4 IN VITRO CONVERSION OF CORTISOL AND CORTISONE

A stock solution of cortisol was prepared by taking the weighed amount of 200 µl of dimethyl sulphoxide and diluting with medium 199 to give 50 µmol 1^{-1} stock solution. This substrate was further diluted into medium 199 plus 20 per cent newborn calf serum and added to coverglass monolayers of the primary explant or cell line to give a final concentration of 500 nmol Cortisol 1^{-1}. Normal culture media were decanted at 7 days, cortisol supplemented medium added and the cultures returned to the incubator. The supplemented medium being decanted at 6, 12 and 24 hours for immunoassay.

Each time interval had control cultures which consisted either of cultures heat treated to 80°C for five minutes (CH), or of a mouse fibroblast line, NCTC 929, to assess conversion of cortisol to cortisone by any fibroblast elements present. A control consisting of cell free medium was also included at each time interval.

Frozen sections of the labial gland biopsy and corresponding labial cultures were then stained routinely for the presence of 11 - B HSD as described in Chapter 5 (Figure 36). Reacting cells in the labial cultures were quantified and expressed as a percentage of cells per cell column.

The relationship between the metabolism of cortisol and 11β HSD activity is shown in Table 15. The primary explant cultures, in which there is both the explant and surrounding

TABLE 15

Relationship between supernatant conversion of cortisol to cortisone from human labial gland cultures and the intracellular presence of 11βHSD

Culture	Incubation Time (h)	Number of cultures	Mean residual cortisol	Percentage of 11βHSD
			Concentration (n mol 1^{-1})	positive cells
Primary labial culture	6	n = 4	359.5	15%
	12	n = 4	361.5	23%
	24	n = 6	354.4	25%
Epithelioid cell line	6	n = 3	376.0	54%
	12	n = 2	332.0	48%
	24	n = 4	244.0	60%
C H cultures	6	n = 2	325.0	
	12	n = 2	383.0	
	24	n = 2	370.0	
NCTC 929	6	n = 2	388.0	
	12	n = 2	392.0	
	24	n = 4	354.8	
Cell free medium	6	n = 2	379.0	
	12	n = 2	385.5	
	24	n = 2	382.5	

cellular outgrowth, metabolised added cortisol as measured by reduction in cortisol concentration. The amount of cortisol metabolised was small but did tend to increase with time of incubation of the culture. There may be a complex effect in these experiments in that the percentage of cells containing 11β HSD tended to be greater with increased incubation time in the presence of cortisol. But this increase in ductal cell number, identified histochemically, did not produce a corresponding increase in the metabolism of cortisol even after 24 hours culture incubation. This observation raises questions of enzyme induction and reversibility of the reaction which will be discussed in Chapter 10.

The experiments were repeated in the absence of the explant, using epithelioid cell lines derived from the primary explant cultures. A similar trend was observed in the increase of cortisol metabolism with time (Table 15). Histochemically, ductal cell number was greater but again, no simple direct relationship was found with cortisol metabolised.

Despite the complications of interpretation, the lack of a direct relationship between cell number and cortisol metabolism in these experiments gave a sufficiently clear indication on the question of whether an assay for the product, cortisone, should be developed. It seemed unlikely that such an assay, taken alone or together with the cortisol assay, would unambiguously yield a method for assaying human salivary ductal cell number in a non-invasive manner.

9.5 STABILITY OF 11β HSD WITH DURATION AND TYPE OF CULTURE

The histochemical assay used for the murine studies was found also readily to localise 11β HSD in primary explant cultures of human labial salivary gland.

In primary explant cultures the number of positively reacting cells was found to be in the range of 15-25 per cent of cells present. A positive reaction is not related to

overall cell morphology since positive cells can be epithelioid or fibroblast-like in appearance. These observations are similar to those found for murine major salivary glands.

Cells reacting positively in the human cultures can be demonstrated when the cellular outgrowth first begins and persist in primary explant cultures at least until 21 days. In human epithelioid salivary cell lines a larger number of positively reacting cells (45-60 per cent) are present in the first and second passage . This compares similarly to murine epithelioid cell lines cultured on glass. Cells grown in plastic have to be harvested to demonstrate 11β HSD histochemically as plastic culture dishes dissolve in the fixatives used. The relationship between cell morphology and positive enzyme reaction cannot therefore be commented upon for cultures grown on plastic.

Primary cultures and glass propagated epithelioid cells have 11β HSD present, as summarised in Table 15. There is no direct relationship between cortisol concentration in the supernatants from primary human labial cultures or epithelioid cultures at 6, 12 and 24 hours and the number of ductal epithelial cells present as indicated histochemically by the presence within cells of 11β HSD activity.

9.6 ELECTRON MICROSCOPY OF HUMAN LABIAL SALIVARY GLAND CELLS

Transmission electron microscopy was principally used to search the human cell cultures for desmosomal cell-to-cell contacts.

Approximately 10 per cent of cells made desmosomal contacts with neighbours and thus could be either ductal, acinar or myoepithelial in origin as evidenced by the presence of desmosomes (Figure 37). Some 16 electron microscope cell sections were studied per explant, over 1000 in all. It does seem that the human cultures are very similar in respect of desmosome contacts to the murine cultures.

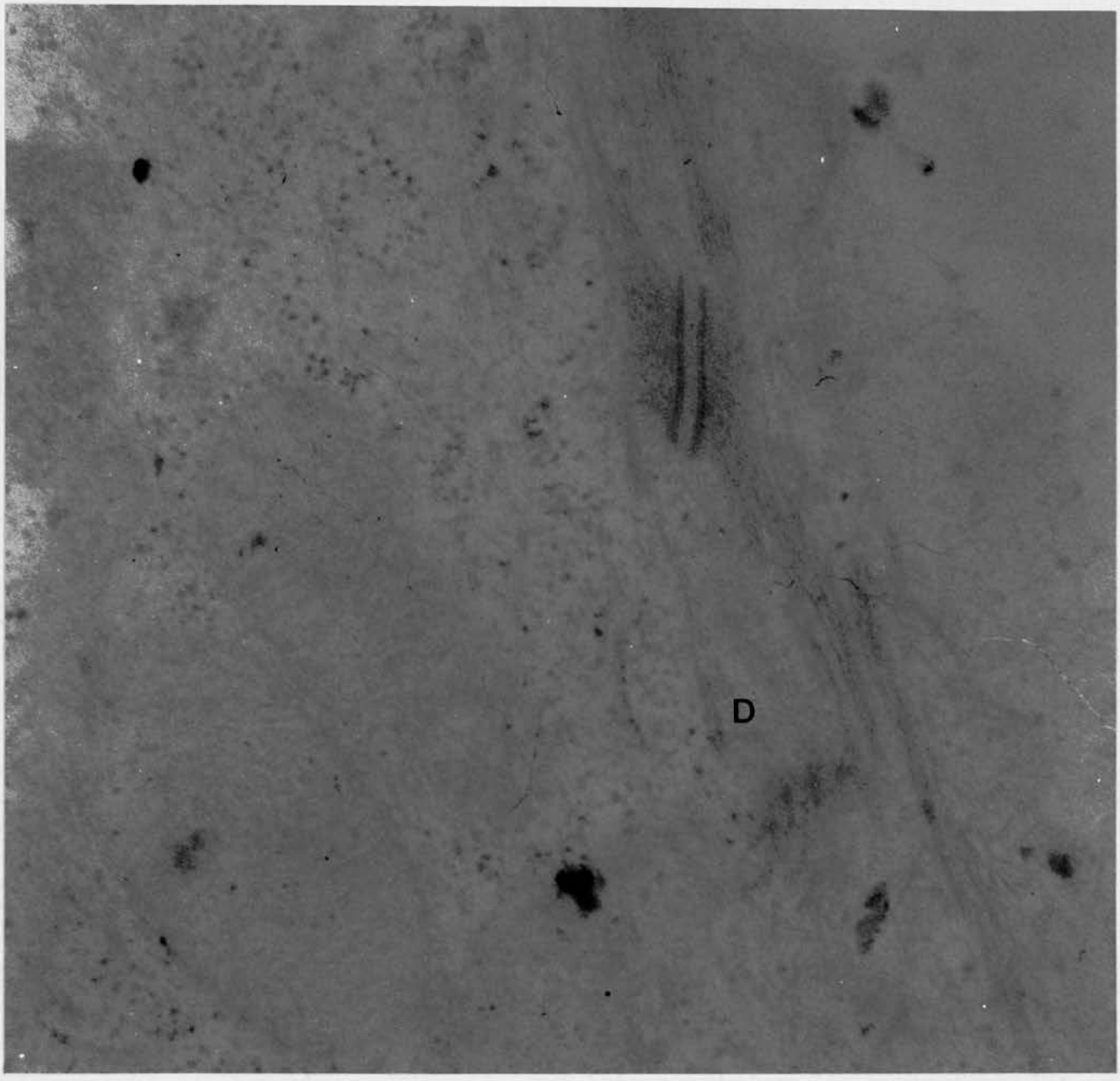


Figure 37

Desmosomal cell-to-cell contact (D) present between adjacent cells in a culture of human labial salivary glands. Such a contact indicates a ductal, acinar or myoepthelial origin for these cells.

X 24,000

9.7 AUTORADIOGRAPHIC STUDIES

The technique employed was that described in Chapter 7 but with the following modifications. Four day old human labial cultures in the log phase of growth were pulsed in normal growth medium containing 1 μCi ml^{-1} ^{3}H thymidine of the same specific activity in that used previously (7.4.1). Cells incorporating tritiated thymidine within the cellular outgrowths were described as being central i.e. within one explant diameter of the explant or peripheral i.e. outgrowth one diameter.

Qualitatively, less thymidine uptake was observed at the periphery of the labial cultures than had previously been observed for adult mouse submandibular gland cultures (Chapter 7). Thymidine uptake at the periphery was not localised in foci but randomly around the periphery of the culture (Figure 35).

9.8 DISCUSSION - IMPLICATIONS OF THE ABILITY TO CULTURE HUMAN LABIAL SALIVARY GLANDS

The current observations have shown that in primary human explant cultures on glass cells are infrequently positive for 11β HSD after approximately 21 days though epithelioid cells may be plentiful. This is similar to the murine cultures. In addition human epithelioid cells cultured on plastic and mechanically harvested show decreasing numbers of 11β HSD positive cells even though cells may be in the 18th passage. One possibility to explain this is that cell shape or substratum contact governs growth of cells (Folkman and Moscona, 1978) ; it could be that cytoskeletal influences are involved not only in growth control but maintenance of a differentiated state, including enzyme expression.

The density of cell growth was less than that seen in adult or newborn mouse submandibular salivary gland. Cultures tended not to grow to confluence. Cell-to-cell contacts are frequently not continuous in the human culture and so a true

comparison of area with respect to cell numbers using only planimetric techniques is not possible. It is not clear why cells derived from human labial gland explants do not utilise all the growth surface available to them. With prolonged culture, the islands of growth are still dominant but streams of fibroblast-like cells develop but these were not positively identified as fibroblasts by the production of collagen or by use of monoclonal antibody (Edwards, Easty and Foster, 1980).

The presence of ultrastructural features such as desmosomes has been taken as evidence of epithelial cell origin (Farquhar and Palade, 1963). Such junctions are also present on myoepithelial cells in sections of human labial salivary glands (Tandler, 1965 ; Tandler, Denning, Mandel and Kutscher, 1970). They are only presumptive evidence of pure epithelial cell types and alone are not a means of differentiating between myoepithelial and ductal cells.

Desmosomal contacts may occupy only 20 per cent of the plasma membrane. The finding of only 10 per cent of cells of the primary human culture or cells of the established human epithelioid cell lines having these junctions, even though histochemically up to 60 per cent of the human epithelial cells are ductal in origin, is open to several interpretations : even though over 1000 serial sections were examined this could be insufficient ; or desmosomal contacts are not maintained <u>in vitro</u> by cells which express such contacts <u>in vivo</u> ; or the enzyme is expressed or active in an unusually large proportion of cells adapted to these <u>in vitro</u> conditions.

In mice the distribution of 11β HSD is purely ductal and thought not to be restricted to any single region of duct. However, in human labial glands intercalated ducts are variable in occurrence and acinar cells which lack 11β HSD may be linked directly to intralobular ducts requiring careful parallel assessment of cell origin in sections used for histochemistry (Tandler et al. 1970). The enzyme was found

in approximately 20 per cent of cells in the outgrowth of primary explant cultures. The question of whether all human ductal epithelial cells contain this enzyme remains to be clarified. Besides the problems of enzyme variance with cellular origin, variations in the cell membrane permeability to the agents of the histochemical reaction will also affect the detection of 11β HSD positive cells.

One way of confirming these observations was to use a radioimmunoassay to quantify cortisol metabolism in the supernatant. No direct relationship could be demonstrated between the amount of cortisol metabolised and the number of cells positive for 11β HSD in either the glass-propagated epithelioid cultures or primary explant cultures.

This study shows that cells having specialised ductal epithelial cell properties can be isolated from labial salivary gland biopsies and can be maintained in culture by repeated passage for over twenty weeks. It proved difficult to maintain an epithelioid cell morphology on coverglass cultures beyond the third passage. Subculture on plastic was successful in maintaining an epithelioid morphology. The ability to grow human salivary cells has opened the way for developing methods for the unambiguous identification, in the human salivary explant outgrowth, of the different cell types identified by classical histology.

CHAPTER 10

GENERAL DISCUSSION AND CONCLUSIONS

Explanations of drug-induced salivary gland enlargement are equivocal owing to lack of detailed information concerning control of normal salivary gland differentiation and growth. Chapter 3 (3.2.3) has alluded to the uncertainty from published work of whether gland enlargement occurs by an increasing number of cells, by enlargement of existing cells, by acquisition or expression of new cell types or a combination of these factors. The interpretive complexities of work on this topic, *in vivo*, clearly require simplification.

The chronic nature of clinical drug-induced salivary gland enlargement also requires an experimental system which will maintain specialised cell types for prolonged periods. Organ culture studies (Malamud and Baserga, 1967) were not adequately described and suffer from the difficulties of measuring cell number and viable cells. Cell dispersion techniques (Kanamura, 1978) have not been extended beyond a few hours *in vitro*.

The usefulness of an *in vitro* technique where individual cell responses require to be analysed depends on achieving sufficient growth for statistical analyses. Therefore, the first problem in the current work was to establish a reproducible system for growing salivary cells *in vitro*. Thereafter, methods of cell identification were required to quantify behaviour of specific cell types. This approach would provide a working system for studying drug-induced responses of properly characterised salivary cells.

In this study conditions were devised to enable small explants of human minor and murine major salivary glands to produce cellular outgrowths *in vitro*. Growth conditions were optimised by studies of explant size, substrata, amount and type of serum supplement, and choice of growth media.

Optimum culture conditions for murine explants of less than 0.4 mm longest dimension, include growth on glass and medium 199 supplemented with 20 per cent (v/v) newborn calf serum, described in Chapter 6.

Cell morphology alone cannot be regarded as a good indicator of cell origin without supporting evidence. In the present work, cells of different appearance were present in the murine explant outgrowth. For convenience, these growing cells were classified by axial ratios as epithelioid or fibroblast-like (Section 7.1).

Accordingly, indices known to be specific for salivary cell populations were applied to the cultured cells. Three indices were used : specialised intercellular contacts detected ultrastructurally ; ductal epithelial cell enzyme 11β hydroxysteroid dehydrogenase ; and the ductal epithelial surface antigen which reacts with a specific salivary duct antibody. The presence of each of these three features enabled cells to be identified more precisely within the cellular outgrowth and are considered good evidence of maintenance of differentiated function in the cultured cells.

Epithelioid cells are generally positive for either the ductal enzyme or for epithelial surface antigen. In addition, the studies demonstrated that some cells positive for ductal epithelial criteria assume a fibroblast-like morphology in vitro. Further, an underestimate of ductal cells by enzyme histochemistry may be due to differential membrane permeability to the components of the histochemical reaction. Other cells although viable by inspection and vital dye exclusion may have reduced enzyme production necessary for the histochemical reaction. The dependence of membrane integrity upon cell viability may result in impaired expression of surface antigens and lead to an underestimate of cell number by the surface antigen technique.

Of the three indices, ultrastructural features were valuable but for practical reasons could not be used to study large populations of cells. The other two indices proved useful routinely and produced comparable values for numbers of ductal epithelial cells in a mixed culture. Recent studies have used additional intracellular antigenic characteristics of human salivary gland cells namely antimitochondrial antibody and smooth muscle antibody. So far the antimitochondrial antibody is too non-specific, but the smooth muscle antibody suggests the presence of myoepithelial cells in mixed cultures (Lamey, Ferguson and Marshall, 1982)

One factor of practical importance is the failure of human salivary gland cells to utilise all the growth surface available. Nevertheless, preliminary studies with human salivary glands were attempted to assess the value of the system by quantifying ductal epithelial cells in a non-invasive manner. The conversion of cortisone by 11β hydroxysteroid dehydrogenase was studied by radioimmunoassay. Conversion of added cortisol was observed but could not be related to the number of ductal epithelial cells present in a culture. The results and controls indicate the factors leading to an underestimate of ductal cells in the histochemical technique, and non-specific cortisol degradation, both contributed to this.

The culture technique finally developed was reproducible, producing cellular outgrowth within predictable limits (Figure 19). Autoradiographic studies confirmed that murine and human cell growth patterns differed. The reasons for this were apparent and were not investigated.

Cultured murine salivary cells with confluent growth characteristics were studied for their response to selected neurotransmitters. Some sympathomimetic agents, principally isoprenaline, produce clinical salivary gland enlargement in humans and mice. This action of isoprenaline was investigated along with other agents to determine if ductal cell proliferation was influenced. The results in Chapter 8 show that none of the neurotransmitters increased the area of

cellular outgrowth from salivary gland explants. Some neurotransmitters inhibited growth, and for isoprenaline at least, inhibition of growth was dose-dependent. Table 9 shows that inhibition was graded from 10^{-4} to 10^{-10}M. This profile is complex. The observed inhibition of growth at high concentration may be a non-specific toxic response to isoprenaline, though the mechanism is unknown. Since inhibition was observed down to 10^{-6}M, it appears isoprenaline may exert some control over salivary cell growth. Nevertheless, no stimulation of growth was observed at any concentration tested.

Table 12 shows that by studies of cell size, the increasing area of cellular outgrowth is not due to cell enlargement. Cell size in each population was found to be unchanged throughout. This finding also confirmed that the inhibitory effect observed was not due to a reduction in cell size collectively reducing the area of cellular outgrowth.

Equally important is the ability to determine which cell types produce the outgrowth and whether neurotransmitters influence the proportion of each type. This was studied by histochemical and cell surface antigen analysis of the cellular outgrowth in response to each neurotransmitter.

Tables 10, 11, 13 show that some agents have an effect principally on ductal epithelial cells. Noradrenaline, acetycholine and 5H-T selectively increase the proportion of ductal epithelial cells. With DOPA there is no alteration in the area of cellular outgrowth and little change in the composition of the cell population. Isoprenaline although producing a dose-dependent inhibition of growth maintains the same proportion of ductal epithelial cells in the outgrowth throughout the dose range 10^{-4}M to 10^{-10}M.

Thus in the cultures, there is a lack of a growth stimulating effect of neurotransmitters which also all alter the proportion of ductal epithelial cells present. This has several possible interpretations.

Firstly, *in vitro* cultures are effectively a denervated system devoid of hormone control which may influence cell proliferation. Secondly, although the epithelial-mesenchymal recombination experiments alluded to in Chapter 1 produced gland growth without requiring hormone supplements the cellular relationships are different in the present work. Here the underlying connective tissue element is no longer providing epithelial cell support nor, possible diffusible factors which affect growth. These factors, may be of fibroblast, acinar or ductal cell origin. The best characterised ductal epithelial cell growth factors are not synthesised in neonatal tissue in significant amounts (Cohen, 1962) but transplacental transfer does occur. Thus a residual effect cannot, from the present studies, be excluded for newborn and foetal calf sera were used as supplements.

Thus the present work has established a method for culturing and identifying salivary gland cells. Use of these cultures for studying drug indiced salivary gland enlargement has shown that neurotransmitters alone neither increase cell number nor cell size. If, as has previously seemed likely from clinical and experimental studies (Borsany and Blanchard, 1961) these components are responsible for salivary gland enlargement, their influence must be mediated indirectly, or requires additional synergistic mediators for expression of enlargement.

Future studies using the present *in vitro* culture approach aim to investigate the role of hormone supplements given concurrently with neurotransmitters whose effects on a specific ductal epithelial cell propulation have now been characterised singly. Since it has now been proven that human salivary cells can be grown, identified and maintained in culture, it is anticipated that human salivary gland explant cultures will allow a study of specialised ductal cell functions to be developed. The study of 11β hydroxysteroid dehydrogenase activity in the human salivary cultures has shown that such studies are feasible and have the advantage of preserving the viable cells studied.

APPENDIX A

Growth requirements of salivary gland explants : gross differences in medium composition

AMINOACIDS	MEDIA				
	199	MEM	TROWELLS	WILLIAMS	WAYMOUTHS
DL. Alanine	+			+(L)	
L. Asparagine				+(L)	
L. Arginine	+	+	+	+	+
DL. Aspartic Acid	+			+(L)	+(L)
L. Cysteine	+		+		+
L. Cystine	+	+		+	+(L)
DL. Glutamic Acid	+				
L. Glutamine	+	+		+	+
Glycine	+			+	+
L. Histidine	+	+	+	+	+
L. Hydroproline	+				
DL. Isoleucine	+	+(L)	+(L)	+(L)	+(L)
DL. Leucine	+	+(L)	+(L)	+(L)	+(L)
L. Lysine	+	+	+	+	+
DL. Methionine	+	+(L)	+	+(L)	+(L)
DL. Phenylalanine	+	+(L)	+	+(L)	+(L)
L. Proline	+			+	+
DL. Serine	+				
DL. Threonine	+	+(L)	+(L)	+(L)	+(L)
DL. Tryptophan	+	+(L)	+(L)	+(L)	+(L)
L. Tyrosine	+	+	+	+	+
DL. Valine	+	+(L)	+(L)	+(L)	+(L)

BALANCED SALT SOLUTION	MEDIA				
	199	MEM	TROWELLS	WILLIAMS	WAYMOUTH
$CaCl_2 2H_2O$		+		+	+
$CaCl_2$	+	+	+		
$CoCl_2 6H_2O$				+	
$CuSO_4 5H_2O$				+	
$FeSO_4$					
KCl	+	+	+	+	+
KH_2PO_4					+
$MgCl_2 6H_2O$					+
$MgSO_4 7H_2O$	+	+	+	+	+
$MgSO_4$					
$MnSO_4 7H_2O$					
NaCl	+	+	+	+	+
$NaHCO_3$	+	+	+	+	+
$NaH_2PO_4H_2O$	+	+	+	+	
Na_2HPO_4			+		+
$(NH_4)_6Mo_7O_{24}4H_2O$					
$ZnSO_4 7H_2O$				+	
$Fe(NO_3)_3 9H_2O$	+			+	
$MnCl_2 4H_2O$				+	

VITAMINS	MEDIA				
	199	MEM	TROWELLS	WILLIAMS	WAYMOUTH
Menadione	+			+	
Nicotinamide	+	+		+	+
Nicotinic Acid	+				
Pyridoxal	+	+		+	
Pyridoxine	+				+
Riboflavin	+	+		+	+
Thiamine	+	+	+	+	+
Vit. A.	+			+	
para-A.B. Acid	+		+		+
Ascorbic Acid	+			+	+
Biotin	+	+		+	+
Vit. B_{12}				+	+
Califerol	+				
D-CaPantothenate	+	+		+	+
Choline Chloride	+	+		+	+
Folic Acid	+	+		+	+
i-Inositol	+	+		+	+
Ergocalciferol (Vit. D_2)				+	
X-Tocopherol Acetate				+	

OTHER COMPONENTS	MEDIA				
	199	MEM	TROWELLS	WILLIAMS	WAYMOUTH
Glucose	+	+	+	+	+
Linoleic Acid				+	
Phenol Red	+	+	+	+	+
Putrescine				+	
Sodium Glutathione				+	
Sodium Pyruvate				+	
Adenine Sulphate	+				
Adenosimetri-phosphate	+				
Adenylic Acid	+				
Tocophenol Phosphate	+				
Cholesterol	+				
Deoxymbose	+				
Hypoxanthine	+				
Ribose	+				
Sodium Acetate	+				
Thymine	+				
Tween 80	+				
Uracil	+				
Xanthine	+				
Guanine	+				
Insulin (bovine)			+		
Dextrose	+				

REDUCING AGENTS	MEDIA				
	199	MEM	TROWELLS	WILLIAMS	WAYMOUTHS
Ascorbic Acid	+			+	+
Glutathione monosodium	+			+	

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A modified autoradiography technique applicable to coverglass cell cultures.

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Subject Interest
CYTOLOGY

SHORT COMMUNICATION

A modified autoradiography technique applicable to coverglass cell cultures.

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As the result of a study of murine salivary gland, it became necessary to develop an autoradiography technique that could be applied to cell monolayers cultured on coverglasses. The objectives of the project were to develop a primary explant technique for the culture of mouse salivary gland cells *in vitro*, and to identify and quantify the cell population present.

Using autoradiography, an attempt was made to quantitate the most prominent areas of cell division within the cultures using 1 μCi/ml of [^{3}H] thymidine as a marker.

The technique devised incorporates the dipping method of Rogers[1] and has the added advantage of limiting the emulsion coating to the side of the coverglass with cellular growth.

Method

1. On completion of the appropriate pulse time the coverglass is removed from the tissue culture medium [Fig. 1(a)] containing the isotope and washed in phosphate buffered saline for a total of 15 min, with changes every 5 min.
2. Cultures are then fixed in 10% formaldehyde in phosphate buffered saline for 12 h.
3. The culture is washed in distilled water for a period of 30 min with at least four changes. Steps 1 and 3 are assisted by gentle agitation.
4. The coverglass cultures are placed on clean filter paper with the cultured surface uppermost and the surplus distilled water allowed to dry off; however, it is important to keep the explant culture and cell surfaces moist with distilled water.
5. A corresponding number of clean dry microscope slides are prepared. This is also a convenient time to record any information or means of identification onto the slide by using a writing diamond.
6. Clear nail varnish is painted onto the slide as a square outlined area corresponding to the size of the coverglass [Fig. 1(b)]. This area is best sited towards one end of the slide as this assists when dipping in emulsion. The coverglass culture is then placed in position with the culture side uppermost. The varnish is allowed to dry (approximately 15–20 min) resulting in adherence of the coverglass to the microscope slide. It is also important that the varnish is allowed to spread to the extreme edge of the coverglass, forming a complete seal to prevent contamination of both sides of the glass by nuclear emulsion when dipping.

It is very important that the culture surfaces are still kept moist with distilled water.

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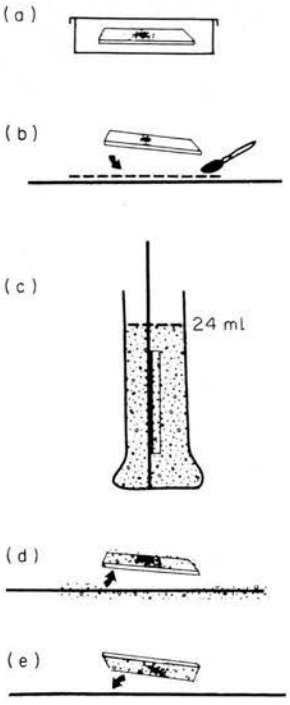

Fig. 1. Diagram showing steps followed for attachment and detachment of coverglass cultures fo dipping technique.

7. Ilford K5 nuclear emulsion[2] is prepared and the dipping technique applied as describe by Rogers[1] [Fig. 1(c)].
8. After drying, the slide cultures are placed in a light-proof box containing silica gel fo the duration of exposure at 4°C. For salivary gland culture the time required was fron 7 to 14 d.
9. On completion of exposure the slides are developed using D19 developer followed b fixing in Kodafix.[3]
10. After washing well in running water for a minimum of 30 min the slide/cultures ar then stained by haematoxylin and eosin.
11. The stained preparations are dehydrated through alcohols and cleared in xylene. Thi was a convenient holding stage when processing a series of cultures.
12. Each slide is then taken individually and placed in acetone to dissolve the varnish bond By applying a sharp razor blade around the periphery of the coverglass detachmen can be hastened [Fig. 1(d)]. Excess varnish should be removed from the coverglas with a paper tissue.
13. Following acetone treatment the coverglass is again dehydrated through absolut alcohol and cleared in xylene.
14. The coverglass with the emulsion coated culture side down is then remounted onto clean dry slide using Kodak Harleco Synthetic Resin[3] [Fig. 1(e)].
15. It is advisable to leave the finished preparations overnight before viewing microscop cally. This allows for proper penetration of the emulsion by the mounting medium.

Results are illustrated in Fig. 2.

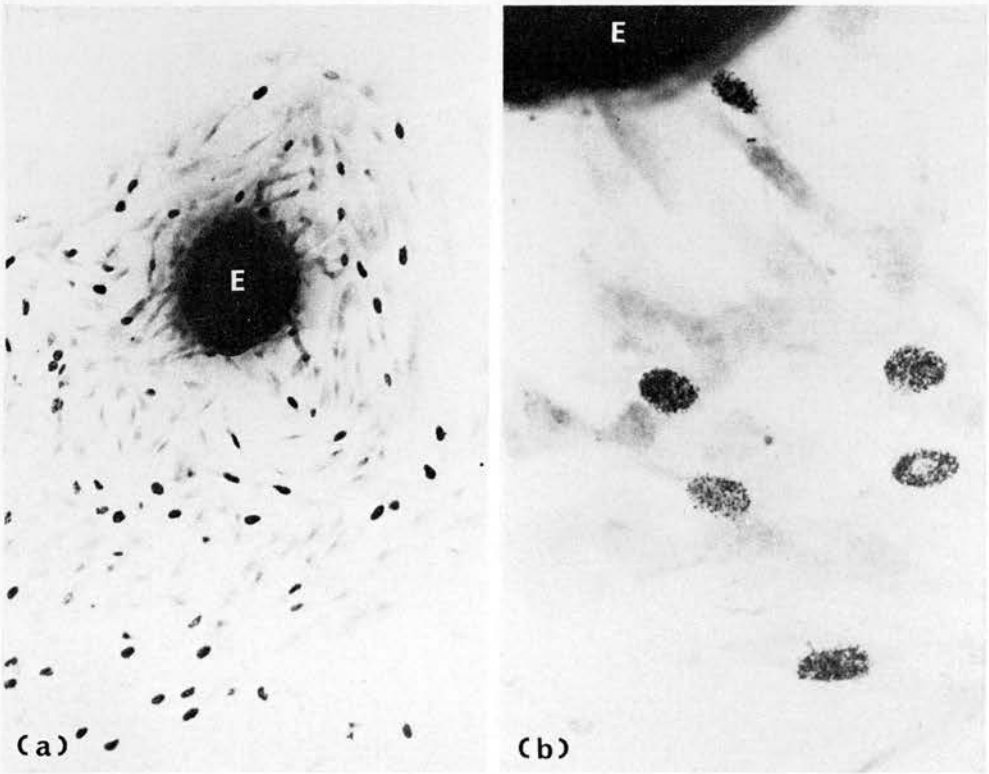

Fig. 2(a) Autoradiograph of salivary gland explant (E) with cellular out-growth. Distribution of positive cells is shown by uptake of [^{3}H]thymidine and is demonstrated by the presence of silver grains (× 60). (b) Periphery of explant (E) with positive nuclei (× 360).

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MURINE SUBMANDIBULAR SALIVARY GLAND *IN-VITRO* GROWTH CHARACTERISTICS UTILIZING CYANOCRYLATE-AIDED ADHESION TO GLASS

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Summary—Primary explants of newborn and adult (CFLP) mouse submandibular salivary gland were cultured using two cyanoacrylate-based materials and a methacrylate-based ultraviolet activated material to produce adhesion to coverglass surfaces. Comparisons were made of adhesion and growth from explants. Identical adhesion was obtained when newborn gland was cultured on coverglasses with or without the cyanoacrylate compounds ethyl alpha cyanoacrylate (EAC) or 3-ethyl cyanoacrylate (3EC) and equal growth occurred when adhesion was direct to glass or when EAC was used. With explants from adult animals, adhesion was enhanced using EAC compared with direct culture and there was no inhibition of growth.

Much tissue culture is concerned with initiating culture growth from discrete fragments of tissue. For some tissues, but not all (New, 1967), it is advantageous to secure the tissue to the substratum until tissue outgrowth anchors the fragments to the chosen surface. Wigley and Franks (1976) referred to this in respect of adult mouse submandibular salivary gland culture. The choice of substratum may be influenced by practical considerations; e.g. polystyrene surfaces dissolve in the solvents involved in the dehydration steps of routine staining procedures. When substrata are needed which will resist solvent attack, for example electron microscopy, cytochemistry or autoradiography (Marshall, Lamey and Ferguson, 1980) glass provides a solvent-resistant substratum which supports good adhesion and growth of salivary gland explant cultures.

Methods of achieving adhesion of explant that appear in the literature include holding the explant in place with a thin film of cellophane (Rose *et al.*, 1958) or by allowing the explant to dry on the surface prior to addition of medium (Levi-Montalcini and Seshan, 1973). However, neither of these studies involved salivary glands.

Submandibular salivary glands from 15 male neonatal and 5 adult male CFLP mice were separated from the adjacent sublingual and parotid glands along fascial planes with the aid of a dissecting microscope and removed aseptically. Following incubation of the entire gland at 37°C in 199 medium (Earle's modified salts, L-glutamine and 15 mmol/l $NaHCO_3$, Gibco Biocult, Glasgow) containing penicillin, streptomycin and mycostatin for 20 min, the glands were cut with fine curved scissors to produce explants with a largest diameter of no more than 0.4 mm.

Thirty-millimetre Petri dishes containing sterile coverglasses (Chance 22 × 22 mm) were prepared with each containing 2.0 ml of 20 per cent newborn-calf serum (Gibco Biocult) in 199 medium. Four explants of uniform size were placed on each coverglass immediately before addition of the medium. In two separate experiments, 48 explants were used for each trial of direct culture alone and 48 explants for each of the two cyanoacrylate compounds, Permabond 102 (Staident Products Ltd, England), main constituent ethyl alpha cyanoacrylate (EAC), and Loctite (U.K. England), main constituent type 3-ethyl cyanoacrylate (3EC). Twenty-four explants were used with the methyl methacrylate/ultraviolet light (wavelength 366 nm) activated material (Nuvaseal, Amalgamated Dental Company) and for the effect of ultraviolet light alone for the one minute needed for Nuvaseal activation. The dishes were kept at 37°C in a humidified atmosphere of 5 per cent CO_2:95 per cent air and the experiment stopped 4 days later. Previous trials showed that all newborn submandibular gland tissue that was going to grow had already done so after 2 days, and the adult tissue after 3 days. Cultures were viewed using an inverted phase-microscope. Adhesion of explant was considered successful if after 4 days it was still attached to its original position and was not freely mobile. Growth was considered to have occurred if cellular outgrowth from the explant was seen. The results analysed using the Chi-square test for significance are summarized in Tables 1 and 2.

The difference between newborn and adult glands with respect to attachment rate and overall growth disappeared when EAC-aided adhesion was used, indicating that toxic products diffusing into the vicinity of the explant were not a problem. However, there was a significant reduction in growth when explants were attached with either Nuvaseal or 3EC. This is apparently not prejudicial to the efficacy of cyanoacrylate compounds *in vivo* (Chou, 1977). Nuvaseal produced greater adhesion than ultraviolet light alone but no growth occurred with Nuvaseal.

Table 1. The effect of varying adherents on the attachments and growth of newborn mouse submandibular gland explants *in vitro*

Group	Adherent	Explants attached	Explants growing following attachment	Overall growth (irrespective of attachment)
A	Direct culture	39/48 (81)	15/39 (38)	15/48 (31)
B	Nuvaseal	18/24 (75)	0/18 (0)	0/24 (0)
C	UV light alone	11/24 (46)	3/11 (27)	3/24 (13)
D	Permabond (EAC)	44/48 (92)	15/44 (34)	15/48 (31)
E	Loctite (3EC)	44/48 (92)	2/44 (5)	2/48 (4)
	Analysis	A:C $p < 0.01$	A:E $p < 0.001$ D:E $p < 0.01$	A:C $p < 0.1$ A:E $p < 0.001$ D:E $p < 0.001$

* Percentages in parentheses.

Table 2. The attachment and growth achieved by direct culture and cyanoacrylate aided adhesion of adult and newborn mouse submandibular gland explant *in vitro*

Group	Tissue	Adherent	Explants attached	Explants growing following attachment	Overall growth (irrespective of attachment)
A	Adult	Direct culture	31/48 (65)	6/31 (19)	6/48 (13)
B	Adult	Cyanoacrylate	43/48 (90)	13/43 (30)	13/48 (27)
C	Newborn	Direct culture	39/48 (81)	15/39 (38)	15/48 (31)
D	Newborn	Cyanoacrylate	44/48 (92)	15/44 (34)	15/48 (31)
Analysis			A:B $p < 0.01$ A:C $p < 0.1$	A:C $p < 0.1$	A:B $p < 0.1$ A:C $p < 0.05$

* Percentages in parentheses.

The experiments show that, where an explant of newborn tissue is itself capable of adhesion, neither of the techniques described appear to improve growth or adhesion. On the other hand, where explant adhesion or organ adhesion is difficult to achieve, EAC improves both adhesion and growth.

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A QUANTITATIVE STUDY ON GROWTH AND CELL POPULATION IDENTIFICATION IN MURINE SALIVARY GLAND CULTURE

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Summary—Differentiation of neonatal salivary gland cell populations was studied *in vitro* using primary explant cultures. Growth on glass of the submandibular, sublingual and parotid salivary glands was optimal if the initial explant diameter was less than 0.4 mm and culture was in 199 medium plus 20 per cent newborn-calf serum. Growth was measured by planimetry of the area of cellular outgrowth as a monolayer with continuous cell contacts. Duct epithelial cells identified using ultrastructural, histochemical and immunological criteria, produced evidence of altered morphology by *in-vitro* conditions. Preservation of enzymic and antigenic markers of ductal epithelial cell origin *in vitro* irrespective of gross cell morphology allowed quantification of these cells in a mixed cell population.

INTRODUCTION

The growth and differentiation of salivary glands is under the complex regulations of both humoral and neural stimuli. To elucidate the role of individual factors on these processes, it is desirable to develop a simplified model system. Using tissue culture techniques, a number of potential mediating factors can be investigated independently. Culture of adult rodent salivary glands has been reported employing both organ culture (Tapp, 1967; Lucas, Peakman and Smith, 1970) and cell culture (Kreider, 1970; Gallagher, Marsden and Robards, 1971; Mercante, 1973; Wigley and Franks, 1976). Certain fundamental problems remain when considering cell culture of salivary glands. These include determining the tissue of origin of the cells and studying the role of one cell type in differentiation. As culture of untransformed epithelial cells alone has frequently been difficult to achieve, a more satisfactory approach may be to accept the non-homogenicity of a culture and identify specific epithelial cell populations present.

The control of tissue behaviour is best studied when some degree of differentiation has been initiated. However, fetal tissue is, in general, more successfully cultured (Trowell, 1959). The salivary glands of the neonatal mouse where the acini are just developing from the ductal epithelium (Yohro, 1970) are a useful source of partly differentiated tissue. Before the effect of any agent on proliferation and differentiation can be evaluated, reproducible conditions of growth in culture must be achieved including explant size, substrata and growth medium.

In primary explant culture the cellular outgrowth arises as a monolayer extending radially from the initial gland explant. Conventional automated counting techniques are not applicable and individual cell counts require cultures to be removed from the incubator for unacceptably prolonged periods. In a monolayer where continuous cell contacts obtain and the original tissue explant size remains a constant, a change in total outgrowth area reflects cell numbers. Measurements of these areas can be conveniently recorded by planimetry. Sequential examination of living cultures facilitates statistical analysis by reducing variance.

Our purpose was to develop a technique for the quantification of cellular proliferation in primary explant cultures and to elucidate the regulatory effects of variable culture conditions. By identifying epithelial cell populations within the outgrowth, the role of chemical mediators upon both salivary gland proliferation and differentiation may subsequently be achieved.

MATERIALS AND METHODS

Dissection and tissue culture techniques

Forty-five neonatal male CFLP mice were killed under ether anaesthesia and all three pairs of major salivary glands were immediately removed with the aid of a dissecting microscope. Routine histology was performed to verify identification. For comparison of adult differentiated tissue, glands from 18 7-week-old male mice were obtained. The whole glands were temporarily retained in 199 medium buffered with 2 per cent Hepes until being finely chopped with curved scissors into explants. Cuboidal explants were prepared ranging in diameter from 0.2–1.00 mm.

Two millilitres of growth medium (199 plus 20 per cent newborn-calf serum, both Gibco Biocult, Paisley, Scotland) was added to each 30 mm plastic Petri dish (Nunclon Nunc, Denmark) and the culture maintained in a CO_2 incubator with a humidified atmosphere of 5 per cent CO_2, 95 per cent air at 37°C. To enable histochemical and immunological comparison between other cell types, standard continuous cell lines of epithelial cells, HeLa, and a mouse fibroblast cell line, NCTC 929, were cultured until confluent under identical culture conditions. A human fibroblast-like cell line was obtained from the lamina propria of a gingival biopsy. The biopsy was excised from

its epithelial component and subjected to repeated trypsinization (Gibco Biocult, trypsin 0.25 per cent) until a cell line with a fibroblast-like morphology (lamina propria culture) was achieved.

Substratum

Six substrata were investigated for their ability to permit growth from primary explants of murine salivary glands and for the ease of preparation for electron microscopy. Primary explants were placed on: (a) glass coverslips cleaned in a non-foaming detergent (Alconox Inc., New York); (b) carbon-coated glass coverslips; (c) poly-1-lysine-coated glass coverslips; (d) Thermanox (Lux Scientific Corporation, U.S.A.) and (e) Melinex, (I.C.I., Welwyn Garden City, England).

The glass slides were coated with carbon at a thickness of 0.1 μm after the method of Kay (1967). Poly-1-lysine (Sigma Chemical Company) was added to the slide with a pipette from a solution containing 75 mg per 100 ml water (Mazia, Schatten and Sale, 1975) and the film allowed to air dry.

Four explants of 0.2–1.0 mm largest diameters were placed on each substratum and incubated in medium 199 plus 20 per cent newborn calf serum for 7 days with the growth medium renewed on day 3. On the 7th day, any cellular outgrowth which had occurred was measured. All cultures were then prepared for transmission to electron microscopy using the epoxy embedding method of Nopanitaya *et al.* (1977) and the ease with which processing could be carried out was assessed.

Medium

Five media (all Gibco Biocult) were chosen and their effect on salivary gland growth evaluated. The media were 199 (Earle's modified salts, L-glutamine and 15 mmol/l $NaHCO_3$), MEM (Earle's salts and L-glutamine), William's (medium D), Trowell's (medium T8) and Waymouth's (medium MB752/1). An arbitrary supplement of 20 newborn calf serum was used initially.

Serum

The sera tested for the neonatal gland cultures were newborn calf, fetal calf, horse (all Gibco Biocult) and mouse. The mouse serum was obtained by bleeding 6-week-old male CFLP mice. Each serum was used at a concentration of 10, 20 and 30 per cent in medium 199.

Bovine and equine sera from three separate commercial batches were used in each trial and results given are the pooled results of 3 separate experiments to reduce possible inter-batch variation.

Quantification

In order to compare the effect of conditions on growth, primary explant cultures of neonatal glands were maintained for 7 days and those from the adult animal for 14 days. A Leitz inverted-phase projection system was used to examine the cellular outgrowth from individual explants in a chronologically longitudinal manner. This approach allows simultaneous visualization of the explants with their surrounding cellular outgrowth and the drawing of these areas. The technique has the advantage of being rapid, reproducible and allows permanent records of growth to be made whilst maintaining continued viability of the explant and the cellular outgrowth from it. This method was particularly useful for quantification of salivary gland growth in which the cellular outgrowth extends radially around the explant and within which cell contacts are continuous (Plate Fig. 1).

The results for attachment and growth were analysed statistically using the chi-squared test and where appropriate, Fisher's exact probability test. In the morphological immunological and histochemical quantification studies, an accumulative means test (Chalkley, 1943) was carried out in order to ascertain the number of cells in both categories which required to be measured to obtain a representative sample. For these studies, a column of cells, approximately 4 cells wide, was chosen extending from the explant centrally out to the periphery; the parameter under investigation was expressed as a percentage.

Light microscopy

To determine whether cell morphology at the light microscopic level would be of value in establishing cell origin in the cellular outgrowth, photographs of all cultures both in the growing and in the stained state were examined, using a Leitz phase microscope. Alternatively, cultures were rinsed in phosphate-buffered saline, fixed in 10 per cent formol saline and stained: haemotoxylin and eosin were used routinely to stain all cultures.

To investigate the relationship between gross cell morphology and other criteria described, an arbitrary division of cell populations was made, because quantitative criteria for identifying cell populations morphologically is lacking. Cells were described arbitrarily as epithelioid (E) if the shortest cell diameter was greater than two-thirds of the longest cell diameter i.e. rounded and (F) if the shortest cell diameter was less than one-third of the longest cell diameter, i.e. spindle-shaped. This classification was used only to provide some quantitation of cell morphology and allow comparison between cells. In Text Fig. 2, cells with a given diameter are presented diagrammatically, thus allowing the population distribution of cells to be seen and analysis for kurtosis carried out.

Electron microscopy

Cultures grown on glass coverslips were removed using the method of Moore (1975) employing hydrofluoric acid dissolution of the coverglass. The lower surfaces of the cultures were then embedded in Araldite and vertical sections through the cellular outgrowth made. Ultra-thin sections were stained with uranyl acetate and lead citrate (Reynolds, 1963) and viewed on a Philips 301 transmission electron microscope. Monolayer cellular outgrowths were examined for specialized cell contacts known to be characteristic of epithelial cells, namely desmosomes with associated tonofilaments (Farquhar and Palade, 1963).

Histochemistry

The enzyme 11β-hydroxysteroid dehydrogenase (11βHSD) is specifically located in ductal epithelial cells (Ferguson, 1967; Ferguson, Glen and Mason, 1970) and would be expected to aid cell identification in mixed cell cultures. Localization of 11βHSD (E.C.

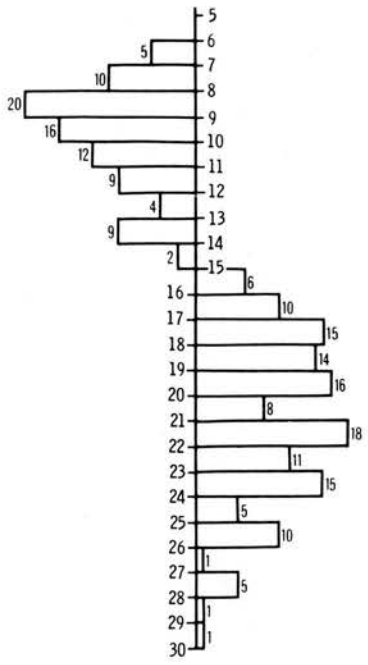

Fig. 2. Frequency distribution of cells with a given unit diameter. The population distribution is bimodal without overlap and without kurtosis.

1.1.1.51.) activity was after the method of Baillie *et al.* (1965), using neonatal mouse submandibular gland cultures and confirming the ductal specificity of this enzyme on frozen sections of these glands (Plate Fig. 3).

Succinate (E.C. 1.3.99.1.), isocitrate (E.C. 1.1.1.42.), glucose-6-phosphate (E.C. 1.1.1.49.) dehydrogenase and arylsulphatases (a and b, E.C. 3.1.6.1.) were demonstrated using standard techniques (Pearse, 1972), and α-ketoglutarate (E.C. 1.2.4.2.) (Ferguson, 1966) was incubated concurrently in a medium without substrate and, upon completion of the incubation, the sections were washed in buffer and mounted using glycerol jelly. Sections were viewed immediately after mounting. Although not specific to one cell population, these enzymes were investigated to determine whether quantitatively their distribution could be used to further identify the epithelial cell population.

Autoradiography

Tritiated thymidine (Radiochemical Centre, Amersham, England) was used at 0.0378 MBq ml^{-1} and of specific activity 1.85 TBq ml^{-1}, pulsed for 6 h periods up to 24 h on the 4-day-old cultures of neonatal mouse submandibular gland. The technique has already been described (Marshall, Lamey and Ferguson, 1980). Standard criteria for cell labelling were adopted (De Robertis, Nowinski and Saez, 1948).

Immunofluorescence

Sera obtained from subjects with Sjögren's syndrome who were proven to be strongly positive for circulating salivary duct antibody was used. This antibody reacts specifically with ductal epithelial cells in a non-species-specific manner. Plate Fig. 4 shows the reaction obtained for human submandibular gland.

Seven-day cultures of neonatal mouse submandibular gland grown on cover-glasses were incubated with undiluted sera at 20°C for 30 min, then rinsed in two changes of 0.5 M barbitone buffer mounted in a buffered glycerol medium and immediately photographed on a Leitz incident u.v. light microscope. Appropriate controls were included.

RESULTS

The submandibular and sublingual glands were readily identifiable. Although gross dissection of the diffuse parotid gland was feasible, histological examination showed that gland samples were inevitably associated with some adipose tissue. Acini were present in all three major glands. Any cellular outgrowth from the adult or neonatal salivary gland explants had occurred after 3 days.

Effect of variable explant size

Of 100 explants, 96 per cent from which cellular outgrowth occurred, were in the range of 0.2 to 0.4 mm in diameter. The remainer which produced outgrowth were in the range 0.4 to 0.6 mm; there was no growth when explant diameter was greater.

Adhesion success rates of 80 per cent were achieved for neontal explants and 70 per cent for adult explants after 7 days in culture. There was no correlation between explant size and adhesion ability.

Effect of various substrata

Adhesion success rates and the area of cellular outgrowth achieved by neonatal submandibular salivary gland explants on each substratum are summarized in Table 1. The use of Thermanox as a substratum permitted maximum cellular outgrowth, but this substratum was impractical in circumstances where exposure to the alcohol dehydration steps following staining procedures is necessary. Direct culture on glass surfaces required chemical dissolution with hydrofluoric acid to remove the Araldite-embedded culture. Although coating the glass with carbon or poly-1-lysine permitted its physical separation with Araldite, such coating seriously impaired cell growth.

Effect of different media

Growth only occurred in the presence of serum.

Text Figure 5 shows the results in respect of growth of neonatal submandibular explants. Similar results were obtained for sublingual and parotid glands. The relative area of outgrowth varied with different media in the decreasing order of 199, William's, Trowell's and MEM; no growth occurred in Waymouth's medium from this source and no other source was tested. At 7 days, all results between individual glands in different growth media are highly significant except between medium 199 and William's medium on the neonatal sublingual glands. Different media thus affect the ability of serum to permit cell growth and division but without apparently influencing explant attachment to the substrata.

Effect of serum concentrations from different species

The results of varying the newborn calf serum concentration in medium 199 between 10, 20 and 30 per

Table 1. Comparison between the effect of different substrata on neonatal mouse submandibular salivary gland explants (0.4 mm maximal diameter) in medium 199 with 20 per cent newborn-calf serum

Substratum	Mean area (units)^2	Explants attached, as percentage initiated
A Alconox-washed glass	320	80 (n = 28)
B Carbon-coated glass	20	50 (n = 30)
C Poly-1-lysine-coated glass	80	58 (n = 26)
D Thermanox	400	80 (n = 28)
E Melinex	300	58 (n = 26)

cent are shown in Text Fig. 6. A 20 per cent supplement of newborn calf serum was optimal.

The effect of different sera is shown in Text Fig. 7. No growth occurred in fetal calf serum. Accordingly, medium 199 with 20 per cent newborn calf serum was used routinely for subsequent cultures. Little inter-batch variation between bovine and equine sera was observed.

Quantification

The planimetric method of quantification is reproducible to within 2 per cent on areas of up to 100 planimetric units^2 (550 planimetric units = 4 mm^2).

Cell morphology at the light microscopic level

The mixed cell populations present in the cellular outgrowth diagramatically represented in Fig. 2 consist of two morphologically distinct cell populations. The group which under the classification outlined previously are referred to as epithelioid have a largest cell diameter of 24 μm, whilst those described as fibroblast-like have a largest cell diameter of 57 μm. This arbitrarily defined morphological classification included 95 per cent of cells.

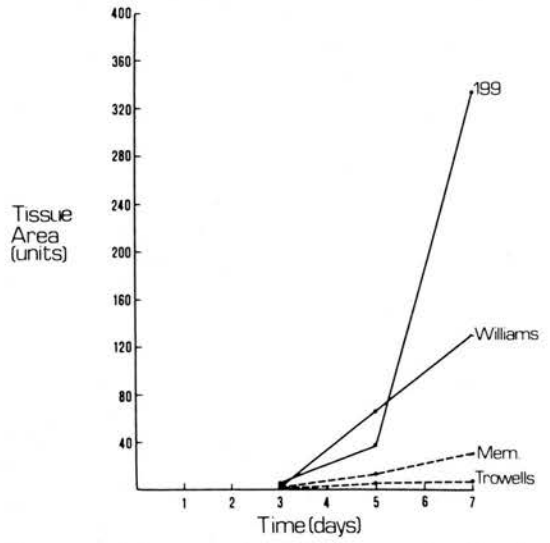

Fig. 5. Graph demonstrating the mean growth achieved at 3, 5 and 7 days by explants of newborn male mouse submandibular gland in various media supplemented with 20 per cent newborn-calf serum.

Epithelioid cells were scattered either singly or in groups throughout the cellular outgrowth and showed no statistically significant predilection for the region immediately adjacent to the explant.

There were no other useful identifying features at the light microscopic level. The epithelioid cells from the mouse cultures had an oval nucleus and several distinct nucleoli. No obvious granules were present in the cytoplasm although vacuoles were occasionally noted. The fibroblast-like cells also had oval nuclei but these cells had characteristic cytoplasmic extensions. No myoepithelial cells as previously described in rat salivary gland cultures were seen (Konisberg, 1960; Cutler and Chaudhry, 1972).

Cell morphology at the electron microscope level

Desmosomes were recognized as regions of cell contact in which the cell membranes of adjacent cells contained a central electron-dense lamina and intra-

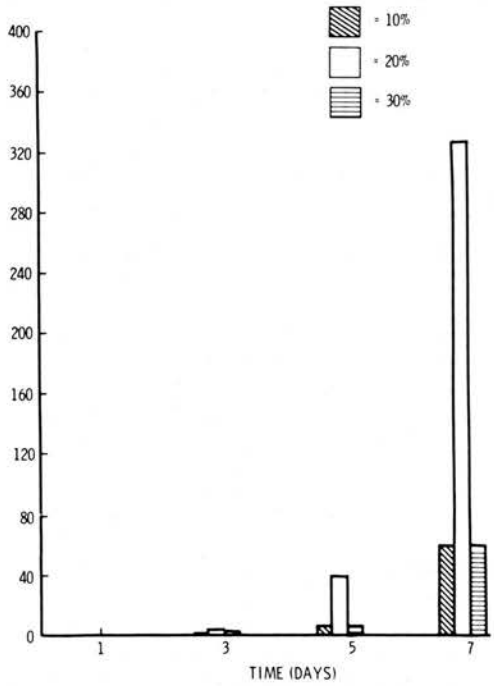

Fig. 6. Histogram demonstrating the effect of varying newborn calf sera concentrations in media 199 on newborn-mouse submandibular gland growth.

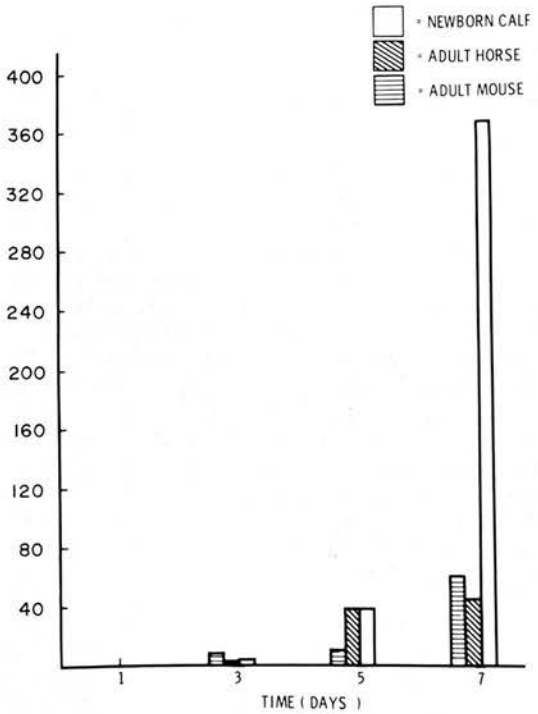

Fig. 7. Histogram demonstrating the effect of varying species serum (20 per cent) in media 199 on newborn-mouse submandibular gland growth.

cellular tonofilaments (Plate Fig. 8). Hemidesmosomes were not observed. No quantification of the number of desmosomal contacts was attempted; subjectively, approximately 10 per cent of cells examined had such specialized cell contacts.

Histochemistry

Table 2 outlines the results of the *in vitro* histochemical reactions carried out on 7-day-old cultures of neonatal mouse salivary gland explants and on monolayers of the established cell lines. With isocitrate dehydrogenase, the reaction was in the form of dense purple granules in the cytoplasm of some cells and appeared, as did the arylsulphatase reaction, to give two cell populations; one reacting strongly and the other weakly. Arylsulphatase activity was predominantly located in the epithelioid cells. 11β-Hydroxysteroid dehydrogenase gave intensely stained granules in the mouse parotid salivary and submandibular glands only (Plate Fig. 9). The reaction was found in cells scattered throughout the growth as well as immediately adjacent to the explant.

Autoradiography

Using a 12 h pulse of 0.037 MBq ml^{-1} [H^3]-thymidine on day 4 of a neonatal mouse submandibular gland culture, there was more cell division at the periphery of the cellular outgrowth than in the region adjacent to the explant.

Immunofluorescence

Plate Fig. 10 illustrates the reaction obtained with salivary duct antibody in culture of neonatal mouse

Table 2. Histochemical results showing absence (−), presence (+), or equivocal (±) reaction for each enzyme

Culture	Succinate dehydrogenase	Isocitrate dehydrogenase	Glucose-6-phosphate dehydrogenase	αKeto glutarate dehydrogenase	11β-Hydroxysteroid dehydrogenase	Arylsulphatase (A + B)
Submandibular gland	+	+	+	±	+	+
Sublingual gland	+	±	+	+	±	±
Parotid gland	+	+	±	±	+	±
HeLa	+	±	+	±	±	±
HCTC 929	+	±	+	±	±	±
Lamina propria	+	±	±	±	±	+

submandibular gland. The reaction in frozen sections was highly specific for the ductal component of the gland; *in vitro* not all the cells giving a positive reaction were epithelioid. This suggests that ductal epithelial cells were present in the cellular outgrowth and *in vitro* can have an epithelioid or fibroblast-like morphology.

DISCUSSION

The ultrastructural observations suggest that central cell viability in explants of less than 0.4 mm in diameter was maintained for at least one week. Initial explant size was therefore a critical factor in sustained explant viability and smaller than that used by Wigley and Franks (1976). Culture of the salivary gland explants produced either cellular outgrowth and the explant assumed a spherical form, or alternatively no cellular outgrowth occurred and in this case the explant had a flat appearance (Plate Fig. 11). These flat explants maintained viability with residual ductal and acinar architecture persisting; this pattern of differentiation was never observed in explants producing a cellular outgrowth.

It is a common observation that culture on plastic surfaces frequently produces growth greater than on glass. This qualitative finding has been substantiated quantitatively for explant cultures on a series of substrata. However, it is known that complex inter-relationships exist between the ability of cells to adhere to the substratum and to the influence of serum growth factors which govern cell shape and growth control (Feldman and Wong, 1977; Greenberg, Grove and Cristofalo, 1977; Folkman and Moscona, 1978).

The choice of medium is reported as affecting the ability of serum to permit cell proliferation and differentiation but not apparently influencing attachment to the substratum. The statistically significant differences in growth achieved by the explants from the same gland receiving the same serum supplement, point to fundamental differences between media.

The growth-promoting activity of serum has been reported by Clarke and Stoker (1971) as showing little species specificity. Our study indicates that there are substantial inter-species variations in the serum growth-promoting property but suggests that species specificity is absent from newborn-calf serum. There appears to be an optimal serum concentration of 20 per cent which, if exceeded, results in growth inhibition.

The methods of quantification for both the measurement of overall growth produced and of cell populations appear to be both valid and accurate if used on monolayer cultures with continuous cell contacts. In conjunction with the autoradiographic procedures these studies do not agree with the originally held concept that cell flattening and migration are the means by which growth of a culture are achieved (Willmer, 1958).

Gross cell morphology can be altered by changes in the cell environment (Folkman and Moscona, 1978). Even in established monoclonal cell cultures, morphology is not constant and therefore morphology alone is an unreliable criterion for cell identification. The epithelial cell line, HeLa, contained a proportion of fibroblast-like cells and the converse was true for both the human lamina-propria fibroblast line and the established NCTC929 mouse fibroblast line. Cultures from all three mouse salivary glands contained both types of cells in varying proportions. Epithelioid cells were scattered throughout the cellular outgrowth including the region immediately adjacent to the explant.

There were no other useful identifying features at the light microscopic level. The presence of two apparently morphologically distinct populations as identified by the frequency distribution of cells with a given diameter indicates that, if more than two cell populations are present, they are presumably morphologically indistinguishable from epithelioid or fibroblast-like cells by the present classification. The bimodal distribution of cells almost certainly does not reflect the distribution of epithelial and mesenchyme-derived cells in the outgrowth. This conclusion is substantiated when the relationship between cell morphology and a positive reaction to 11βHSD or to salivary duct antibody is considered. Some cells reacting with salivary duct antibody had an epithelioid and others a fibroblast-like morphology as in Fig. 10.

The presence of ultrastructural features characteristic of one cell type provides a simple aid to cell population identification independent of gross cell morphology. Only the desmosomes were assessed; it is the best known junction of epithelium and tight junctions have been shown to vary in some tissues with both the type of dehydration agent used (Johnston and Koots, 1967) as well as the staining procedure (Brightman and Reese, 1969). The criteria for identification of desmosomes is well established (Odland, 1958; Stern, 1965).

Ultrastructural features such as desmosomes with 7 nm tonofilaments found in a proportion of cells in the cellular outgrowth from the salivary gland explants are indicative of epithelial cell origin. The practical disadvantage of this means of identification as the only evidence of cell origin is that it is time consuming and does not readily show the overall position of the cell with these contacts in the cellular outgrowth. In addition, because in some species desmosomes in non-keratinized oral epithelium (Chen, 1970) occupy less than 20 per cent of the plasma membrane, there is a probability of missing such attachments unless serial sections are examined.

Histochemistry allows cell identification on the basis of function in unfixed cells. There is a possibility of differences of epithelial and fibroblast membrane permeability to components of the histochemical reaction. A considerable advantage of this method is that it alllows localization of epithelial cells in relation to the overall cellular outgrowth as well as allowing their numbers to be counted.

In salivary glands, 11β-hydroxysteroid dehydrogenase is located in the ducts (Ferguson, 1967; Ferguson *et al.*, 1970) where it is responsible for the conversion of cortisol to cortisone (Ferguson and MacPhee, 1975). Hence the location of this enzyme in the cells of the outgrowth is consistent with their ductal origin.

Other histochemical reactions may also be of value in cell identification. The negative reaction for isocitrate dehydrogenase in fibroblast cell lines may be useful in conjunction with other tests. Fresh sections of mouse submandibular gland stain intensely for succinic dehydrogenase (Ferguson, 1966) predominantly

in the intra-lobular duct epithelium. In culture, intense cell staining reaction was also found for this enzyme in some cells. Duwey (1958) believed that the distribution of succinic dehydrogenase accords well with that of mitochondria; in rat parotid, duct cells showed marked activity whereas acinar cells reacted only moderately.

Arylsulphatase, whilst not giving a reaction specific for either cell type, appeared to distinguish two cell populations. The work of Kostulak (1977) suggests that the detection of this enzyme could be a useful adjunct to epithelial cell identification. None of the other enzymes investigated appeared to be of much value in cell-type determination.

The conclusion from our study that the epithelial component in the cellular outgrowth from primary explants of neonatal mouse submandibular and parotid gland is ductal in origin, is in contrast with work which suggests that the epithelial component is acinar in origin (Kreider, 1970; Gallagher *et al.*, 1971).

A salivary duct antibody has been described in rheumatoid arthritis (Bertram and Halberg, 1964; MacSween *et al.*, 1967). With an indirect immunofluorescence technique the cytoplasm of the salivary gland ducts stain specifically. This immunological reaction and the principle of its use is under-used in the field of tissue culture (Diaz and Marcelo, 1978) although the potential of histochemistry and immunofluorescence as aids to identifying cell populations in culture are out of accord with the view that differentiated epithelium loses its specialization rapidly *in vitro* (Sato, Zaroff and Mills, 1960; Sandstrom, 1965).

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Plate 1.

Fig. 1. Section 1 μm thick vertically through an explant (EX) and a monolayer cellular outgrowth (OG).

Fig. 3. Typical histochemical reaction to 11β hydroxysteroid dehydrogenase in a frozen section of mouse submandibular salivary gland showing the specifically ductal localization of the reaction. $\times 400$

Fig. 4. Reaction to salivary duct antibody in a frozen section of human submandibular salivary gland. $\times 600$

Fig. 8. Transmission electron micrograph of two adjacent cells in the monolayer cellular outgrowth shown in Fig. 1. Three intercellular desmosomes (D) indicate the epithelial origin of the cells.

Fig. 9. Typical reaction obtained to 11β-hydroxysteroid dehydrogenase *in vitro*. The cytoplasmic localization of enzyme activity can be seen in two cells with surrounding negatively stained cells.

Fig. 10. Reaction obtained in cellular outgrowths to salivary duct antibody. A reaction is present in cells of various morphologies. $\times 400$

Fig. 11. A flat organotypic culture. A ductal pattern is discernible with surrounding lobular, presumably acinar, cells. $\times 100$

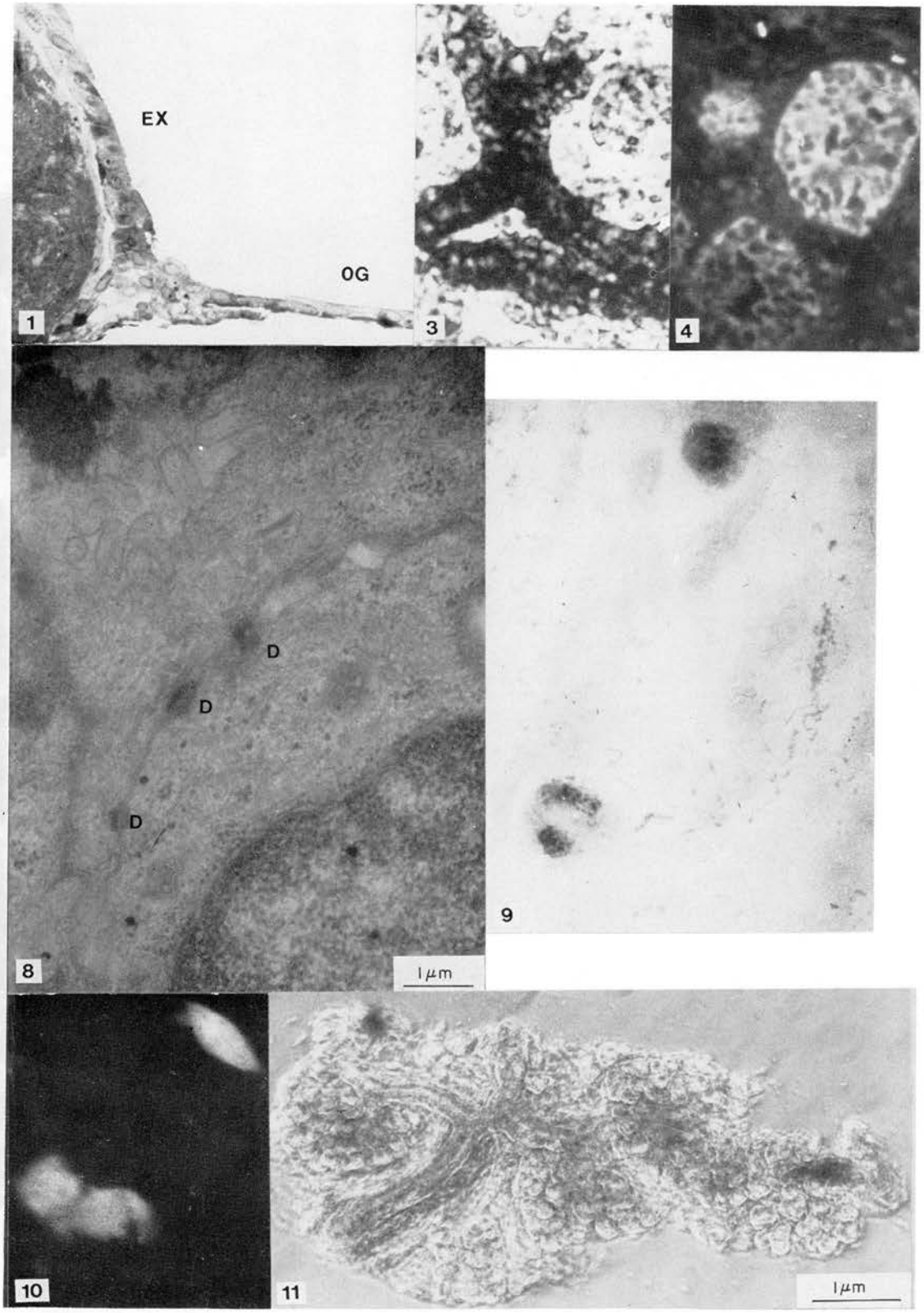

Plate 1.